RECENT INSIGHTS INTO THE DOUBLE ROLE OF HYDROGEN PEROXIDE IN PLANTS

EDITED BY: Naser A. Anjum, Sarvajeet Singh Gill, Francisco J. Corpas, Cristina Ortega-Villasante, Luis E. Hernandez, Narendra Tuteja, Adriano Sofo, Mirza Hasanuzzaman and Masayuki Fujita

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RECENT INSIGHTS INTO THE DOUBLE ROLE OF HYDROGEN PEROXIDE IN PLANTS

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Editorial: Recent Insights Into the Double Role of Hydrogen Peroxide in Plants

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Editorial on the Research Topic

Recent Insights Into the Double Role of Hydrogen Peroxide in Plants

Reactive oxygen species (ROS) of varied types can be yielded in plants at several primary sites (such as the chloroplast, mitochondria, and peroxisomes) under normal aerobic metabolism *via* processes including photosynthetic and respiratory electron transport chains. However, impaired oxidant-antioxidant balance and extreme growth conditions in plants are bound to cause increases in the cellular concentrations of radical and non-radical ROS such as superoxide anions ($O^{2\bullet-}$), hydroxyl radical (OH•), singlet oxygen ($_1O^2$), and hydrogen peroxide (H_2O_2). On the one hand, H_2O_2 has no unpaired electrons and is moderately reactive. Owing to its relative stability compared to other ROS and its capacity for diffusing through aquaporins in the membranes and over more considerable distances within the cell (Bienert et al., 2007), H_2O_2 acts as a stress signal transducer and contributes to numerous physiological functions in plants. On the other hand, H_2O_2 is a relatively long-lived molecule with a half-life of 1 ms, readily crosses biological membranes, and consequently can bring oxidative consequences far from the site of its formation (Neill et al., 2002; Sharma et al., 2012; Sehar et al., 2021). The Frontiers Research Topic "*Recent Insights into the Double Role of Hydrogen Peroxide in Plants*" highlighted the major mechanisms underlying the dual role of H_2O_2 in response to different abiotic stresses in plants. This Research Topic incorporated 19 publications, including 10 original research articles, 8 reviews, and one perspective article.

H_2O_2-METABOLISM AND H_2O_2-PRIMING ROLES IN ABIOTIC STRESS MANAGEMENT

As a potent signaling molecule H_2O_2 gets produced in routine in stressed or non-stressed conditions *via* dismutation of $O^{2\bullet-}$ radicals through superoxide dismutase (SOD) during electron transport in different compartments of the plant

cell, and is involved in the regulation of the plant growth, metabolism, and stress tolerance. It has also been noted that at higher concentrations in the cell during oxidative stress, ROS, including H_2O_2, can oxidize vital biomolecules (like nucleic acids, proteins, and lipids) and significantly impacts the seed germination process (Wojtyla et al.). Among the major abiotic stress factors, several heavy metals provoke increases in the production of ROS through plasma membrane-bound NADPH oxidases. However, the relationship of H_2O_2 has also been established in heavy metal tolerance in crop plants (Cuypers et al.). H_2O_2 directly mediates metal-induced oxidative signaling, where the production of H_2O_2 may involve H_2O_2 receptors, redox-sensitive transcription factors and inhibition of phosphatases (Miller et al., 2008). H_2O_2 sensing in metal-exposed plants also involves activation of mitogen-activated protein kinase (MAPK) pathways (Opdenakker et al., 2012). Additionally, interaction of H_2O_2 with Ca^{2+} (Baliardini et al., 2015), NO (Arasimowicz-Jelonek et al., 2012) and oxylipins (Tamás et al., 2009; Keunen et al., 2013) was also reported in metal-exposed plants. Though excess accumulation of H_2O_2 and polyamines (PAs) can be detrimental for the plant cell leading to premature cell death, a fine-tuning of these signaling molecules (H_2O_2 and PAs) can result in stress management by coordinating intra-cellular and systemic signaling systems (Gupta et al.). Polyamine oxidase (PAO)-induced production of H_2O_2 was found to be involved in the coleorhiza-limited rice seed germination (Chen et al.). ROS-specific probe DCFH2-DA enabled confocal laser scanning microscopy revealed a high level of ROS in the stigma at different developmental stages (unopened flower buds, recently opened flowers, dehiscent anthers, and flowers after fertilization) of scrutinized plants (Zafra et al.).

During evolution, plants have developed an efficient ROS-scavenging system constituting an array of enzymatic (SOD; CAT, catalase; APX, ascorbate peroxidase; GR, glutathione reductase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GOPX, guaiacol peroxidase, and GST, glutathione-S-transferase) and non-enzymatic (AsA, ascorbic acid; GSH, glutathione; phenolic compounds, alkaloids, non-protein amino acids, and α-tocopherols) antioxidants to get rid of excessive ROS in the cell (Singh et al.). Notably, NADH oxidase (RBOH), alternative oxidase (AOX), the plastid terminal oxidase (PTOX), and the malate valve with the malate dehydrogenase isoforms are involved in maintenance of the cellular redox homeostasis under salinity stress (Hossain and Dietz). In *Arabidopsis* cell suspension cultures, anoxia stress/shock led to significant increases in H_2O_2 (and also nitric oxide, NO); however, re-oxygenation maintained the components of ROS scavenging machinery like ascorbate-glutathione (AsA-GSH) system, α-tocopherol, and eventual cell survival as result of decreased H_2O_2 (Paradiso et al.). *Eutrema salsugineum* (halophyte) and *Arabidopsis thaliana* (glycophyte) exhibited a differential pattern of accumulation and scavenging of ROS. In particular, compared to *A. thaliana* chloroplasts, *E. salsugineum* chloroplasts showed a constitutive increase and the cell's steady-state regulation of H_2O_2 level which prepared this plant for ROS-control mainly due to an efficient ROS-scavenging machinery including glucosinolates content and well-coordinated tuning of hormonal signaling (Pilarska et al.). Elevation in the cellular level of H_2O_2 and its consequences can be controlled by brassinosteroids, a class of plant-specific essential steroid hormones. To this end, in tomato seedlings, brassinosteroid (24-epibrassinolide) ameliorated the impacts of zinc oxide nanoparticles-caused elevated H_2O_2 by enhancing the activity of enzyme involved in superoxide-dismutation (SOD), H_2O_2-metabolizing enzymes (catalase, CAT; and APX), increasing GSH-regeneration (as a result of increased GSH reductase activity; and consequently decreasing GSH-oxidation), finally inducing the transcripts of *Cu/Zn SOD*, *GSH1*, *CAT1*, and *GR1* (Li et al.). In a comprehensive *in silico* study, APX and GSH-peroxidase (GPX) genes/proteins from 18 different plant species were identified and compared in order to unravel their significance in excessive H_2O_2 management (Ozyigit et al.). Notably, APX and GPX were found to be involved in the metabolism of antioxidants and secondary metabolites, redox homeostasis, stress adaptation, and photosynthesis/respiration. The major redox proteins namely plant peroxiredoxins (Prxs) and sulfiredoxins (Srxs) are involved in antioxidant defense and redox signaling in stressed plants. Srxs were are also found to be involved in antioxidant defense and redox signaling in response to environmental stimuli; post-translational modifications of Srxs regulate the ROS-transduction and bioactivity. On the other hand, Prxs are sensitive to glutathionylation. Investigation of the glutathionylation of recombinant chloroplastic 2-Cys Prx and mitochondrial Prx IIF of pea plants revealed glutathionylation-mediated change of the decameric form of 2-Cys Prx into its dimeric glutathionylated form. Additionally, the reduced dimeric form of Prx IIF was glutathionylated without changing its oligomeric state (Calderón et al.). Thus, glutathionylation was argued to depend on the GSH/GSSG ratio owing to the perceptible difference in the exact effect on the 2-Cys Prx and Prx IIF proteins.

H_2O_2-priming (exposure of seeds, seedlings, or plants to stressors/chemical compounds that makes them ready to tolerate the later stress events) helps in biotic and abiotic stress tolerance in various crop plants by triggering the ROS scavenging machinery (Dikilitas et al., 2020). Exogenous supply of H_2O_2 can induce stress tolerance under salt, drought, chilling, high temperatures, and heavy metal stress (Hossain et al.). In a study on mustard (*Brassica juncea* L.) cultivars, H_2O_2-induced reversal of the major negative impacts of Ni stress (200 mg Ni kg^{-1} soil) led to increased photosynthetic nitrogen-use efficiency, sulfur-use efficiency, and GSH content and decreased levels of lipid peroxidation and electrolyte leakage (Khan et al.). Notably, H_2O_2 priming-mediated increased tolerance to cadmium-caused oxidative stress in *Brassica napus* involved fine-tuning between the glyoxalase system and the components of ROS-scavenging machinery (Hasanuzzaman et al.).

H_2O_2 CROSSTALK WITH OTHER MOLECULES

Along with H_2O_2, other signaling molecules (such as nitric oxide, NO; and calcium, Ca^{2+}) and phytohormones (such as jasmonic

acid, JA; salicylic acid, SA; and abscisic acid, ABA) play key roles in stress signaling cascades and crosstalk during plants' stress responses (Saxena et al., 2016). To this end, the crosstalk of H_2O_2 with NO and Ca^{2+} was argued to contribute to regulation of the plant development and abiotic stress responses (Niu and Liao). Notably, the role of SA in adventitious root formation involved H_2O_2 acting as a downstream messenger (Yang et al., 2013). Having emerged as a master regulator of stress responses, ABA signaling pathway triggers significant changes in gene expression and plants' adaptive physiological responses (Saxena et al., 2016). There occurs a close relation among the MAPK cascades, ABA, JA, SA, and H_2O_2 where exogenous application of H_2O_2 triggers MAPK cascade, which in turn involves ABA, JA, and SA (Saxena et al.). ABA-induced H_2O_2 accumulation can protect plant parts (such as pumpkin-grafted cucumber leaves) against $Ca(NO_3)_2$ via ABA/H_2O_2 signaling-led induction of ROS-scavenging machinery (Shu et al.). *S*-nitrosoglutathione reductase (GSNOR) determines the level of *S*-nitrosothiol and thereby regulates NO-signaling in plants (Lindermayr, 2018; Jahnová et al., 2019). In *A. thaliana*, H_2O_2 *in vitro* led to inhibition of the activity of GSNOR and significantly changed NO-homeostasis, which in turn resulted in the activation of ROS-scavenging machinery in order to suppress the oxidative damage (Kovacs et al.).

CONCLUSIONS AND FUTURE PERSPECTIVE

In the current Research Topic "*Recent insights into the double role of hydrogen peroxide in plants,*" the contributions discussed the versatile role of H_2O_2 as a signaling molecule that triggers the upregulation of the components of antioxidant defense machinery and imparts tolerance in crop plants against the variety of environmental cues. The crosstalk of H_2O_2 with other signaling molecules and phytohormones leads to signal transduction in response to various stresses and regulates plant growth, development, and stress tolerance. Therefore, further understanding on the coordination of H_2O_2 and other signaling molecules NO, Ca^{2+}, MAPK, SA, and ABA can pave the way to achieving tolerance in crop plants to increasing stress conditions.

AUTHOR CONTRIBUTIONS

NA and SG prepared the first draft of the manuscript. FC, CO-V, LH, NT, AS, MH, and MF read and revised the manuscript. All authors listed approved the final version for publication.

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REVIEW
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Hydrogen peroxide priming modulates abiotic oxidative stress tolerance: insights from ROS detoxification and scavenging

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Plants are constantly challenged by various abiotic stresses that negatively affect growth and productivity worldwide. During the course of their evolution, plants have developed sophisticated mechanisms to recognize external signals allowing them to respond appropriately to environmental conditions, although the degree of adjustability or tolerance to specific stresses differs from species to species. Overproduction of reactive oxygen species (ROS; hydrogen peroxide, H_2O_2; superoxide, $O_2^{\cdot-}$; hydroxyl radical, $OH^{\cdot}$ and singlet oxygen, $^1O_2$) is enhanced under abiotic and/or biotic stresses, which can cause oxidative damage to plant macromolecules and cell structures, leading to inhibition of plant growth and development, or to death. Among the various ROS, freely diffusible and relatively long-lived H_2O_2 acts as a central player in stress signal transduction pathways. These pathways can then activate multiple acclamatory responses that reinforce resistance to various abiotic and biotic stressors. To utilize H_2O_2 as a signaling molecule, non-toxic levels must be maintained in a delicate balancing act between H_2O_2 production and scavenging. Several recent studies have demonstrated that the H_2O_2-priming can enhance abiotic stress tolerance by modulating ROS detoxification and by regulating multiple stress-responsive pathways and gene expression. Despite the importance of the H_2O_2-priming, little is known about how this process improves the tolerance of plants to stress. Understanding the mechanisms of H_2O_2-priming-induced abiotic stress tolerance will be valuable for identifying biotechnological strategies to improve abiotic stress tolerance in crop plants. This review is an overview of our current knowledge of the possible mechanisms associated with H_2O_2-induced abiotic oxidative stress tolerance in plants, with special reference to antioxidant metabolism.

Keywords: hydrogen peroxide, abiotic stress, oxidative stress, priming, stress tolerance

Introduction

In plants the production of reactive oxygen species (ROS) is a common outcome of various metabolic reactions that occur in multiple sites within a plant cell. ROS like hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), the hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2^{\cdot}$) are also produced as one of the earliest responses of plant cells to environmental stresses, and these ROS molecules can cause damage to a variety of biological processes (Halliwell, 2006; Gill and Tuteja, 2010; Das and Roychoudhury, 2014). In plants subjected to various abiotic stresses, such as salt, drought, chilling, heat and metal or metalloid stresses, ROS levels can rise significantly, leading to redox imbalance and oxidative stress (Hossain et al., 2010; Hasanuzzaman et al., 2011a,b; Hossain and Fujita, 2013; Mostofa and Fujita, 2013; Das and Roychoudhury, 2014; Mostofa et al., 2014a,b,c; Nahar et al., 2014). High ROS levels can result in extensive damage to proteins, DNA, and lipids, thereby affecting normal cellular functions, which can lead to permanent metabolic dysfunction and plant death (Anjum et al., 2015). To combat oxidative stress, plants have developed an elaborate system to control cellular ROS titer (Mittler et al., 2011). Surprisingly, plants have also evolved a way to exploit lower titer of ROS as signaling component to regulate wide variety of plant processes, including cell elongation, differentiation, morphogenesis and responses to environmental stress (Dat et al., 2000; Foreman et al., 2003; Tsukagoshi et al., 2010; Bhattacharjee, 2012).

In the last decade, H_2O_2 received considerable interest among the ROS and other oxygen-derived free radicals. H_2O_2, the result of two electron reduction via $O_2^{\cdot-}$ (the first step one electron reduction component), possesses the highest half-life (1 ms) of the ROS. A comparatively long life span and the small size of H_2O_2 molecules permit them to traverse through cellular membranes to different cellular compartments, facilitating signaling functions, including retrograde signaling (Apel and Hirt, 2004; Bienert et al., 2006; Maruta et al., 2012; Noctor et al., 2014). The signaling role of H_2O_2 is well established, particularly with reference to plant processes like stress acclimation, antioxidative defense, cell wall cross-linking, stomatal behavior, phytoalexin production, regulation of the cell cycle, and photosynthesis. So, the toxicity or danger associated with H_2O_2 on one hand and signaling cascades on other make it a versatile molecule whose concentration needs to be tightly controlled within plant cells (Petrov and Van Breusegem, 2012).

There are multiple sources of H_2O_2 in plant cells, including over-energization of electron transport chains (ETC) or redox reactions in chloroplasts or mitochondria, fatty acid oxidation, and photorespiration (**Figure 1**). Of these sources, the most significant is oxidation of glycolate in the peroxisome during the photosynthetic carbon oxidation cycle. In addition, the oxidative burst associated with part of hypersensitive response to pathogens also cause rapid increase in the concentration of H_2O_2 (Miller et al., 2010). One of the main sources of H_2O_2 is a class of cell membrane-bound NADPH-dependent oxidases that are similar to the respiratory burst oxidase homologs (RBOH). In plants, RBOH are in fact enzymes regulated by a class of Rho-like proteins called 'ROP's (Rho-related GTPases; Agrawal et al., 2003). Another class of enzymes, associated with the formation of H_2O_2, is the cell wall-associated peroxidases (Bolwell et al., 2002). Rates of H_2O_2 accumulation in peroxisomes and chloroplasts may be 30–100 times higher compared with H_2O_2 formation in mitochondria. Importantly, ROS formation in mitochondria does not vary significantly in presence or absence of light, since the total O_2 consumption is less affected by light than TCA cycle activity. However, the formation of $O_2^{\cdot-}$ by electron transport systems can be influenced by light, if exposure to light affects alternative oxidase activity (Dutilleul et al., 2003). Alternative oxidases have been found to influence ROS generation and to be involved in determining cell survival under stressful conditions (Maxwell et al., 1999; Robson and Vanlerberghe, 2002).

The antioxidant systems that regulate H_2O_2 levels consist of both non-enzymatic and enzymatic H_2O_2 scavengers. Enzymes, such as catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione *S*-transferases (GSTs), glutathione reductase (GR), and peroxyredoxin (Prx), and non-enzymatic compounds, like ascorbate (AsA), glutathione (GSH), α-tocopherol and flavonoids, are constantly involved in regulating the concentration of ROS, including H_2O_2 (Miller et al., 2010; Kapoor et al., 2015). In fact, both the production and scavenging of H_2O_2 in plant cells seem to be integrated in a network and are responsible for the 'biological effect.' The paradox of H_2O_2 physiology lies with its opposing activities; at higher concentrations it causes oxidative damage to important cellular metabolites, whereas at lower concentrations it initiates cell signaling (Gechev and Hille, 2005; Bhattacharjee, 2012). The redox imbalance associated with environmental stresses, such as salinity and extremes of temperature, increases the overall rate of metabolism and eventually up-regulates H_2O_2 production in plant cells (Bhattacharjee, 2012, 2013).

Priming (pre-treatment of seeds or plants by exposure to stressor or chemical compounds, making them more tolerant to later stress events) is potentially an important mechanism of induced resistance in plants against biotic stresses (Beckers and Conrath, 2007; Tanou et al., 2012; Borges et al., 2014). Recent studies have shown that priming can also modulate abiotic stress tolerance (Filippou et al., 2012; Hossain and Fujita, 2013; Mostofa and Fujita, 2013; Borges et al., 2014; Mostofa et al., 2014a,b,c; Nahar et al., 2014; Wang et al., 2014a). Despite the agronomic and ecological importance of priming, in-depth molecular mechanisms associated with priming in plants are still unknown (Conrath, 2011). Mounting evidence suggests that the initial exposure to chemical priming agents (such as H_2O_2, ABA, NO, SA etc.) renders plants more tolerant to abiotic stresses (Wang et al., 2010a; Hasanuzzaman et al., 2011a; Mostofa and Fujita, 2013; Mostofa et al., 2014a; Sathiyaraj et al., 2014; Teng et al., 2014). A number of studies on plants have demonstrated that the pre-treatment with an appropriate level of H_2O_2 can enhance abiotic stress tolerance through the modulation of multiple physiological processes, such as photosynthesis, and by modulating multiple stress-responsive pathways, such as the ROS and methylglyoxal (MG) detoxification pathways (Azevedo-Neto et al., 2005; Chao et al., 2009; Liu et al., 2010a; Xu et al.,

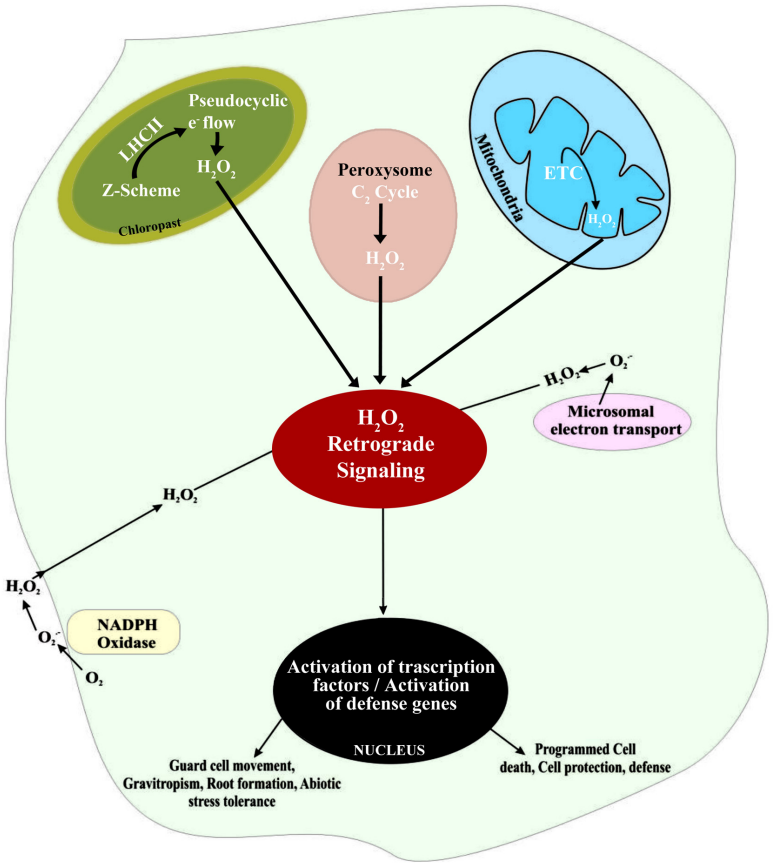

FIGURE 1 | Schematic representation of H_2O_2 generation in different intra- and extra-cellular sites and the subsequent signaling associated with the regulation of defense gene expression in plant cells.

2010; Wang et al., 2010a, 2014a; Ishibashi et al., 2011; Gondim et al., 2012, 2013; Hossain and Fujita, 2013). Although H_2O_2 is known to act as a signaling molecule, activating multiple defense responses that reinforce resistance to various environmental stresses in plants (Petrov and Van Breusegem, 2012), very little is known about the mechanisms by which plants perceive/sense H_2O_2 and how this sensing mechanism is coordinated within the developmental program of a plant. In this review, we summarize our current understanding of the possible mechanisms associated with H_2O_2-induced enhanced abiotic stress tolerance with special reference to ROS detoxifying/scavenging proteins and gene expression.

Exogenous H_2O_2 and Abiotic Stress Tolerance

Many recent studies on plants have demonstrated that H_2O_2 is a key player in the signal transduction process associated with tolerance to abiotic and biotic stresses, and the induction of the stress cross-tolerance phenomena often observed in plants. A number of reports, discussed in more detail below, have shown that exogenous application of H_2O_2 can induce tolerance to salinity, drought, chilling, and high temperatures, and heavy metal stress, all of which cause elevated H_2O_2 production (Gong et al., 2001; Uchida et al., 2002; Azevedo-Neto et al., 2005; Chao et al., 2009; Liu et al., 2010a; Wang et al., 2010a, 2014a; Ishibashi et al., 2011; Gondim et al., 2012, 2013; Hossain and Fujita, 2013).

Exogenous H_2O_2 and Salt Stress Tolerance

The salt stress-induced oxidative burst due to uncontrolled ROS accumulation has been well documented in plants. However, several recent studies on plants have demonstrated that pre-treatment with exogenous H_2O_2 can induce salt tolerance. Uchida et al. (2002) studied the effects of H_2O_2 and nitric oxide (NO) pre-treatments on oxidative stress in rice (*Oryza sativa*) plants under salt or heat stress. Their results showed that seedlings treated with low concentrations (<10 μM) of H_2O_2 or NO resulted in greener leaves and a higher photosynthetic activity than that of the control plants under conditions of salt or heat stress. It was also shown that pre-treatment induced increases in ROS scavenging enzyme activities and increased expression of genes encoding Δ′-pyrroline-5-carboxylate synthase, sucrose-phosphate synthase, and the small heat shock protein 26. Their findings indicate that NO and H_2O_2 act as signaling

molecules that modulate heat and salt stress tolerance by regulating the expression of stress-related genes. In addition, Azevedo-Neto et al. (2005) found that supplementation of the nutrient solution with H_2O_2 induced salt tolerance in maize plants, by enhancing antioxidant metabolism and reducing lipid peroxidation in both leaves and roots. Wahid et al. (2007) reported that exogenous H_2O_2 improved salinity tolerance in *Triticum aestivum* when seeds were soaked in H_2O_2 (1–120 μM, 8 h) and subsequently grown in saline conditions (150 mM NaCl). H_2O_2 levels in the seedlings, arising from H_2O_2-treated seeds, were markedly lower when grown under saline conditions than control seedlings from seeds not treated with H_2O_2, and also exhibited better photosynthetic capacity. These results suggest that seedlings from H_2O_2-treated seeds had more effective antioxidant systems than found in untreated controls. Moreover, the H_2O_2 treatment appeared to improve leaf water relations, helped to maintain turgor, and improved the K^+:Na^+ ratio of salt stressed seedlings. H_2O_2 treatment also enhanced membrane properties, with greatly reduced relative membrane permeability (RMP) and lower ion leakage. Surprisingly, the expression of two heat-stable proteins (32 and 52 kDa) was also observed in H_2O_2 pre-treated seedlings. Fedina et al. (2009) reported that *Hordeum vulgare* seedlings pre-treated with H_2O_2 (1 and 5 mM) had higher rates of CO_2 fixation and lower malondialdehyde (MDA) and H_2O_2 contents, following exposure to 150 mM NaCl for 4 and 7 days, when compared with seedlings subjected to NaCl stress only. In addition, the leaf Cl^- content of NaCl treated plants was considerably less in H_2O_2 pre-treated plants. The above findings indicate that H_2O_2 metabolism might be important for the induction of salt tolerance.

Gondim et al. (2010) evaluated the roles of H_2O_2 on the growth and acclimation of maize (*Zea mays*) triple hybrid (BRS3003) seedlings exposed to salinity stress, with three consecutive studies. In the first studies, H_2O_2 accelerated the percentage germination of seeds at 100 mM, but not at 500 mM H_2O_2. In second study, pre-treatment of seeds with H_2O_2 caused an up-regulation of APX and CAT activities after 30 h. In contrast, GPX activity was lower in seeds primed with H_2O_2 for 12, 24, 30, 36, and 42 h as compared with the seeds primed with water only. The activity of SOD was not affected by pre-treatment of seeds with H_2O_2, except for the 24 h pre-treatment. In the third experiment, seeds were pre-treated by soaking in 100 mM H_2O_2 for 36 h, or in distilled water (DW), and then grown in a culture solution with or without salt stress (80 mM NaCl). Their findings showed that priming of seeds with H_2O_2 increased seedling tolerance to salinity, with seedlings demonstrating improved growth rates. The differences in the levels of antioxidant enzyme activities detailed above may explain the higher salinity tolerance of seedlings from seeds pre-treated with H_2O_2. In addition, Li et al. (2011) reported that exogenously applied H_2O_2 (0.05 μM) reduced the MDA content, enhanced the GSH content and increased the activities of APX, CAT, SOD, and POD in wheat seedlings under salt stress. A similar response in *Suaeda fruticosa* (a halophyte) was also found, indicating that cellular defense antioxidant mechanisms are enhanced by the exogenous application of H_2O_2 (Hameed et al., 2012). Up-regulation of the activities of CAT and SOD following the exogenous application of H_2O_2 (0.5 mM) was also observed in oat (*Avena sativa*) plants under salt stress (Xu et al., 2008). Similarly, Gondim et al. (2012) found that foliar H_2O_2 priming was effective in minimizing salt stress in maize and analysis of the antioxidant enzymes CAT, GPOX, APX, and SOD revealed that the H_2O_2 foliar spray increased the activities of all of these enzymes. CAT was found to be the most highly responsive of the above enzymes to H_2O_2, with high activities observed (48 h) after treatment, while GPX and APX responded much later (240 h after treatment). Lower MDA levels were also detected in maize plants with higher CAT activities, which may have resulted from the H_2O_2 detoxifying function of this enzyme. In addition, Gondim et al. (2013) studied the influence of exogenous H_2O_2 application on AsA and GSH metabolism, relative chlorophyll content, relative water content (RWC), and gas exchange, in *Zea mays* grown under salinity. Photosynthesis and transpiration, stomatal conductance, and intercellular CO_2 concentrations all declined in plants under salt stress; however, the negative impact of salt stress was not as great in plants sprayed with H_2O_2. In addition, H_2O_2-sprayed plants had higher RWCs, relative chlorophyll contents and lower leaf H_2O_2 accumulation, which correlated positively with improved gas exchange, compared with control plants under conditions of NaCl stress. The non-enzymatic antioxidants AsA and GSH did not appear to play any obvious roles as ROS scavengers in this study. The authors of the above study concluded that salt tolerance of maize plants, brought by pretreatment of leaves with H_2O_2, was due to less H_2O_2 accumulation and to maintenance of the leaf RWC and chlorophyll contents. These characteristics allowed higher photosynthesis and improved growth of maize plants under salt stress. In addition to these findings, Ashfaque et al. (2014) conducted an experiment to study the role H_2O_2 played in mitigating salt stress in wheat (*Triticum aestivum* L.) plants. Treatment of plants with H_2O_2 positively influenced plant growth under saline and non-saline conditions. The application of 50 or 100 μM H_2O_2 reduced the severity of salt stress, with reductions in both Na^+ and Cl^- ion levels and an increase in proline content and in N assimilation. Improved water relations, increased levels of photosynthetic pigments and greater growth rates were also observed in H_2O_2 under salt stress when compared with untreated plants. Under non-saline conditions application of H_2O_2 also improved all the parameters detailed above. Treatment with 100 μM H_2O_2 provided maximal protection for wheat plants grown under non-saline conditions and also alleviated the effects of salt stress in plants grown under saline conditions. Recently, Sathiyaraj et al. (2014) found that *Panax ginseng* seedlings treated with 100 μM H_2O_2 for 2 days showed enhanced salinity tolerance and increased activities of APX, CAT, and guaiacol peroxidase. Other oxidative parameters such as MDA levels and endogenous H_2O_2 and $O_2^{\cdot-}$ levels were lower in H_2O_2 treated salt-stressed seedlings. Seedling dry weight, and chlorophyll and carotenoid contents were also greater in H_2O_2 treated seedlings than in untreated controls, when seedlings were subjected to salt stress. The above findings demonstrate that H_2O_2 priming can induce tolerance to salinity in plants by modulating physiological and metabolic

processes such as photosynthesis, proline accumulation and ROS detoxification, and that this ultimately leads to better growth and development. Importantly, ROS metabolism also plays a pivotal role in the development of stress and cross stress tolerance.

Exogenous H_2O_2 and Drought Stress Tolerance

Drought stress is widely thought to induce oxidative stress by increasing the levels of H_2O_2 and singlet oxygen (de Carvalho, 2013). However, Jing et al. (2009) investigated the capacity of H_2O_2 priming to promote drought tolerance in Cucumber plants. Drought stress resulted in cucumber plants with round chloroplasts, and indistinct chloroplast membranes and thylakoids. While H_2O_2 priming did not change chloroplast ultrastructure, priming did increase the activities of the antioxidant enzymes SOD, CAT, GPOX, APX, DHAR, DHAR, GR, and the levels of AsA and GSH, resulting in lower levels of MDA, H_2O_2 and $O_2^{\cdot-}$. The authors of this study concluded that by increasing antioxidant capacity H_2O_2 priming reduced the accumulation of ROS in treated plants, and alleviated some of the membrane damage found in the chloroplasts of plants under drought stress. In a similar study Ishibashi et al. (2011) showed that spraying plants with H_2O_2 could alleviate the symptoms of drought stress in soybean. The RWC content, photosynthetic rate and stomatal conductance of drought-stressed leaves in plants sprayed with H_2O_2 were all higher than in leaves sprayed with DW. In contrast to spraying with DW, spraying with H_2O_2 caused an increase in the expression of *galactinol synthase* (*GolS*) and *d-myo-inositol 3-phosphate synthase 2* (*GmMIPS2*) genes, which are responsible for the synthesis of oligosaccharides. These findings indicated that H_2O_2 spraying enabled soybean plants to avoid drought stress by helping to maintain leaf water levels, and that leaf water retention was probably due to increased oligosaccharide biosynthesis rather than rapid stomatal closure. Abass and Mohamed (2011) also studied the effects of priming seeds with H_2O_2 on the drought tolerance of common bean seedlings (*Phaseolus vulgaris* L.). A significant decrease in plant growth parameters, photosynthetic pigments, and the total carbohydrate content was observed in response to drought stress. In contrast, a significant increase in compatible solutes, polyamine and antioxidant levels, and abscisic acid (ABA) contents were observed in plants in response to drought stress. H_2O_2-priming of seeds enhanced all of the above parameters in seedlings grown under drought conditions when compared with the seedlings of water-treated seeds. The above findings suggest that H_2O_2 could trigger the activation of defense mechanisms, including increased levels of antioxidants, which then persist in developing seedlings and help to alleviate damage and improve plant growth and performance under drought. Liao et al. (2012) studied the beneficial roles of exogenous NO and H_2O_2 in marigold (*Tagetes erecta* L.) adventitious root formation in response to drought. NO or H_2O_2 treatment reduced the damage to mesophyll cell ultrastructure caused by drought stress. NO or H_2O_2 treatment also increased leaf chlorophyll contents, chlorophyll fluorescence parameters (Fv/Fm, ΦPS II, and qP), and hypocotyl soluble carbohydrate and protein contents, while reducing starch contents. These findings demonstrate that NO or H_2O_2 can protect mesophyll cell ultrastructure from damage, improve the photosynthetic performance of leaves and mitigate the negative effects of drought stress, by enhancing nitrogen and carbohydrate accumulation. Recently, Hossain and Fujita (2013) examined the potential biochemical mechanisms of H_2O_2 priming-induced drought tolerance in mustard (*Brassica juncea* L.) seedlings by investigating ROS scavenging and MG metabolism. Eight-day-old seedlings were pre-treated with a low concentration (50 μM) of H_2O_2 for 24 h prior to the imposition of drought stress for 48 h. H_2O_2 priming enhanced cell membrane stability in leaf tissues under drought stress, by reducing tissue MDA contents. The levels of endogenous H_2O_2, in H_2O_2 pre-treated, drought stressed-seedlings were markedly lower than that of seedlings subjected to drought stress without H_2O_2 pre-treatment. Lower activities of APX, CAT, and Gly II were observed in response to drought stress, whereas DHAR, GPX, and Gly I activities significantly increased. AsA, GSH, and GSSG levels increased significantly, whereas the GSH/GSSG ratio decreased in drought-stressed seedlings. Surprisingly, H_2O_2 pre-treated drought-stressed seedlings maintained significantly higher APX, GR, CAT, GST, and Gly II activities, as well as a higher GSH/GSSG ratio compared with seedlings under drought only. These results show that H_2O_2 priming can activate both ROS and MG detoxification pathways and modulate the tolerance of seedlings to water deficit (Hossain and Fujita, 2013). Ashraf et al. (2014) investigated the beneficial roles of exogenous H_2O_2 on drought stress tolerance in maize. Maize seedlings were pre-treated with different concentrations of H_2O_2 and grown under conditions of water stress. Higher germination percentages were found in seeds soaked in 140 mM H_2O_2. Drought led to a sharp decrease in photosynthetic pigments, whereas the levels of H_2O_2, lipid peroxidation and AsA increased. The activities of CAT, SOD, and POX rapidly increased. Importantly, the 140 mM H_2O_2 treatment reduced photosynthetic pigment degradation and lipid peroxidation and increased the activities of antioxidant enzymes and AsA levels. The beneficial influence of exogenous H_2O_2 treatments have also been observed in plants under osmotic stress. Liu et al. (2010a) studied the effects of exogenous H_2O_2 on osmotic stress-induced alterations in the ultra-structures of chloroplasts and mitochondria in two cucumber (*Cucumis sativus* L.) varieties. Osmotic stress caused the degradation of chloroplast and mitochondrial membranes in both cucumber genotypes and increased MDA levels. Osmotic stress and exogenous H_2O_2 both increased MnSOD, GPX, CAT, GPOX, APX, GR, MDHAR, DAHR activities and levels of the antioxidants AsA and GSH. The combined effects of osmotic stress and exogenous H_2O_2 resulted in the highest antioxidant levels in both cucumber ecotypes. Liu et al. (2010a) proposed that pre-treatment with H_2O_2 increased antioxidant levels in the leaves of cucumbers, thereby decreasing MDA levels, and protecting the ultrastructure of most chloroplasts and mitochondria in plants under osmotic stress. Terzi et al. (2014) also found that exogenous H_2O_2 (10 mM) pre-treatment induced osmotic stress tolerance in maize (*Zea mays* L.) seedlings. H_2O_2 treatment caused a decrease in MDA levels and stomatal conductance, whereas an increase in endogenous H_2O_2, leaf water potential, ABA

concentration, and metabolite levels, including soluble sugars, proline, and polyamines, were observed. Osmotic stress caused a decline in leaf water potential and stomatal conductance, but the levels of MDA, H_2O_2, metabolite levels and the ABA content increased. Importantly, H_2O_2 pretreated osmotical stressed seedlings showed improved water status and stomatal conductance, as well as accumulation of MDA, H_2O_2, ABA, and metabolites. These results demonstrate that H_2O_2 pre-treatment induces osmotic stress tolerance by increasing soluble sugar, proline, and polyamine levels.

Exogenous H_2O_2 and Chilling Stress Tolerance

The positive role of exogenous H_2O_2 in modulating low temperature stress tolerance has been well documented. Prasad et al. (1994a,b) reported that addition of H_2O_2 modulated chilling tolerance, due to a transient increase in H_2O_2-activated acclimation mechanisms. The authors suggested that H_2O_2 has dual effects on maize plants during acclimation to chilling; it serves as a signal to induce the synthesis of ROS-scavenging enzymes, and in non-acclimated seedlings it accumulates to higher levels and acts as a destructive agent. Additionally, it was reported that both H_2O_2 and SA could mediate the induction of protective mechanisms against abiotic stresses. SA pre-treatment induced an increase in H_2O_2 concentrations that in turn triggers an increase in antioxidant enzyme activities and eventually leads to higher tolerance to chilling stress in maize seedlings (Janda et al., 1999). Likewise, H_2O_2 and SA were involved in the signal transduction pathway leading to acclimation during heat stress in mustard (Dat et al., 1998). Yu et al. (2003) showed that a transient oxidative shock, induced by exogenous H_2O_2, effectively increased chilling tolerance in mung bean (*Vigna radiata* L. cv. V3327) seedlings. Seedlings pre-treated with 200 mM H_2O_2 had increased survival rates (from 30 to 70%) and lowered EL (86 to 21%). Importantly, the endogenous level of H_2O_2 was not affected by exogenous application of H_2O_2. Surprisingly, exogenous H_2O_2 repressed the stimulation of ROS detoxifying enzymes APX and CAT; however, GSH levels increased significantly under both chilling and control conditions. Pre-treatment of mung bean plants with both ABA and H_2O_2 showed no synergistic effect on GSH content. The authors concluded that H_2O_2-mediated chilling tolerance in mung bean plants might be mediated by an increase in GSH content that is independent of ABA. Supporting this finding, Hung et al. (2007) showed that H_2O_2 pre-treatment induced chilling tolerance in chilling sensitive mung bean seedlings (*V. radiata* L. Cv Tainan Number 5). Seedlings pre-treated with 200 mM H_2O_2 or cold-acclimated (10°C for 48 h in the light) showed lower electrolyte leakage (EL) compared to seedlings subjected to chilling stress (4°C for 36 h) without H_2O_2 treatment or cold-acclimation. Chilling tolerance induced by H_2O_2 appeared to depend on the accumulation of GSH, as tolerance could be reversed by pretreatment with buthionine sulfoximine (BSO). In contrast, tolerance induced by cold-acclimation was neither accompanied by the accumulation of GSH nor reversed by BSO, suggesting that there are at least two independent mechanisms for developing chilling tolerance. Wang et al. (2010a) studied the effects of foliar pre-treatment with H_2O_2 in modulating chilling stress tolerance of mascarene grass (*Zoysia tenuifolia*) and manilagrass (*Zoysia matrella*). Pre-treatment with H_2O_2 (10 mM) was found to modulate chilling (7°C/2°C, day/night) stress tolerance as indicated by lower MDA and EL levels and higher protein contents. Pre-treatment significantly increased the activities of APX, GPX, and CAT in *Zoysia matrella* and APX, GR, and POD activities in *Zoysia tenuifolia*, indicating that H_2O_2 acts as a signaling molecule and modulates the metabolic responses associated with ROS-induced damage caused by chilling. Importantly, optimal pre-treatments reduced any increases in H_2O_2 levels, improved chilling tolerance, and increased CAT, POD, APX, GR, and GPX activities. Therefore, antioxidative enzymes are likely to be important factors for the acquisition of chilling tolerance in both *Zoysia* cultivars. Moussa and Mohamed (2011) showed that priming of pea seeds with H_2O_2 or NO significantly enhanced drought induced oxidative stress tolerance. Seeds were pre-treated with 70 mM H_2O_2 or 10 μM sodium nitroprusside (a NO donor). Seeds pre-treated pea seedlings have less ROS-induced damage, accelerated proline synthesis and enhanced total chlorophyll and carotenoid contents, increased photosynthetic activity, and increased growth when subjected to osmotic stress. Drought stress reduced the activities of APX, GPX, and CAT, and caused an overproduction of $O_2^{\cdot-}$ in the leaves of pea plants, which in turn increased MDA levels and reduced photosynthetic performance. Pre-treatment with SNP or H_2O_2 modulated the activities of antioxidant enzymes, limited $O_2^{\cdot-}$ production, and inhibited membrane peroxidation under drought stress, which indicated an enhanced operation of antioxidant systems. Moreover, after H_2O_2 or SNP pre-treatment seedlings had enhanced membrane stability as revealed by a lower MDA contents. The increased production of antioxidants in seedlings from seeds pre-treated with H_2O_2 or SNP persisted for some time, alleviating ROS-induced impairment and modulating the physiological characters associated with drought tolerance of seedlings. Recently, İşeri et al. (2013) investigated whether exogenous H_2O_2 application could influence the short-term cold responses of tomato plants and induce acclimation. Pre-treatments were performed by immersing roots into 1 mM H_2O_2 solution for 1 h and then transferring the seedlings to the soil (acclimated group). Cold stress (3°C for 16 h) caused a significant reduction in the RWC of control and non-acclimated groups when compared with unstressed plants. H_2O_2 promoted the maintenance of a higher RWC under stress. Anthocyanin levels in the leaves of acclimated plants under cold stress were significantly higher than those of unstressed control and non-acclimated plants. High MDA levels demonstrated low temperature induced oxidative damage in control and non-acclimated plants. MDA levels in acclimated plants remained similar to those of unstressed plants, which demonstrated that the H_2O_2 acclimation process protected the cells against cold induced lipid peroxidation. In addition, H_2O_2 acclimation caused proline accumulation in roots under cold stress and APX activity in the roots of cold-stressed and -unstressed H_2O_2-acclimated plants increased when compared with control and non-acclimated plants, with the highest increase in the roots of acclimated plants under cold stress. CAT levels in

the roots of acclimated plants also increased, whereas levels remained unchanged in unstressed plants. Endogenous H_2O_2 levels increased significantly in the roots of control and non-acclimated plants under cold stress. In contrast, the H_2O_2 content of the roots of acclimated plants was significantly lower than that of control and non-acclimated plants under cold stress. These results demonstrate that H_2O_2 significantly enhances oxidative stress responses by elevating the antioxidant status of tomato plants.

Exogenous H_2O_2 and Heat Tolerance

Like other abiotic stresses endogenous levels of H_2O_2 increase in heat stressed plants (Hossain et al., 2013a,b; Mostofa et al., 2014b) and exogenous pre-treatments haves been found to increase the heat tolerance of plants. Kang et al. (2009) reported that H_2O_2 pre-treatments increased the activities of APX and glucose-6-phosphated dehydrogenase (G6PDH) in cucumber and tomato seedlings, and induced tolerance to heat stress. Bhattacharjee (2012) reported that H_2O_2 enhanced tolerance of two rice cultivars differing in salt tolerance (SR 26B, salt-tolerant; Ratna, salt-sensitive cultivar) to heat- or chilling-induced oxidative stress. Salt or drought stress results in significant increases in lipid peroxidation and protein oxidation, along with concomitant increases in the accumulation of ROS ($O_2^{\cdot-}$ and H_2O_2) and a reduction in antioxidant defenses (assessed in terms of total thiol content and the activities of SOD, CAT, APX, and GR) in both the seedlings of salt-sensitive Ratna and salt-tolerant SR 26B cultivars. Imbibitional treatment with low concentrations of H_2O_2 reduced oxidative damage to newly assembled membrane systems caused by heat and chilling stress in the seedlings of both cultivars of rice (Ratna and SR 26B). Imbibitional H_2O_2 treatment also caused an increase in antioxidant defenses (activities of SOD, CAT, APX, GR, and total thiol content) in the heat and chilling stressed seedlings and caused a significant improvement in the early growth performances of both cultivars. Better responses to H_2O_2-mediated acclamatory performances and restoration of redox-homeostasis under extremes of temperature were noted for the salt-sensitive rice cultivar Ratna compared with the salt-tolerant SR 26B. In general, these results suggest a significant role for an 'inductive pulse' of H_2O_2 in acclimatizing plants to adverse temperatures, by helping to maintain redox-homeostasis and mitigating oxidative membrane, protein and lipid damage during the recovery phase of the post-germination event. Wang et al. (2014a) studied the beneficial roles of exogenous H_2O_2 in modulating heat stress tolerance in turfgrass species. Ryegrass (*Lolium perenne* cv. Accent) and tall fescue (*Festuca arundinacea* cv. Barlexas) were sprayed with 10 mM H_2O_2 before they were exposed to heat stress (38/30°C, day/night) and compared with plants maintained at control temperatures (26/15°C, day/night). Before being subjected to heat stress seedlings treated with H_2O_2 were found to have increased activities of POD, CAT, APX, GR, and GPX, as well as larger AsA and GSH pools. Importantly the ratio of GSH/GSSG was also lower. Under heat stress H_2O_2 pre-treated seedlings showed lower oxidative damage and H_2O_2 levels, and increased activities of APX, GR, GST, and GPX. These results indicated that H_2O_2 could up-regulate the antioxidant defense systems that ultimately lead to improved thermotolerance in turfgrass species.

Exogenous H_2O_2 and Heavy Metal Stress

Excessive production of ROS, especially H_2O_2, in response to heavy metal exposure has been widely observed in plants (Hossain et al., 2010; Mostofa and Fujita, 2013; Mostofa et al., 2014a). H_2O_2 priming has also been found to increase tolerance of plants to heavy metals. Hu et al. (2009) showed H_2O_2 pre-treatment induced Cd tolerance in rice (*O. sativa*). Cd stress led to a significant decrease in both the length and biomass of roots and shoots. However, pre-treatment with 100 μM H_2O_2 for 1 day mitigated Cd stress and increased the levels of the antioxidant enzymes (SOD, CAT, GPX, APX, and GST), as well as elevated the levels of GSH and AsA. Consequently, the levels of MDA and H_2O_2 declined and the growth rates of the seedlings improved. H_2O_2 pre-treatment also decreased the Cd concentration found in the shoots, and lowered the shoot:root Cd ratio, indicating that H_2O_2 may affect Cd distribution in rice seedlings. Improved Cd tolerance was thought to be partly due to enhanced antioxidant metabolism that effectively prevented the increase in ROS levels under Cd stress. Higher Cd sequestration in root tissue may also contribute to the decline in Cd translocation. Chao et al. (2009) investigated the role of GSH in modulating heavy metal (Cd) stress tolerance of rice seedlings. Seedlings treated with either a heat shock or H_2O_2 showed a significant increase in leaf GSH levels. Treatment with exogenous GSH under non-heat stress conditions, which also resulted in an increase in GSH levels in leaves, also enhanced the Cd tolerance of rice seedlings. Pre-treatment of seedlings with an inhibitor of GSH synthesis inhibited the increase in GSH levels caused by heat shock or H_2O_2 treatment and caused a reduction Cd tolerance. Importantly, the negative effects of BSO could be reversed by the addition of GSH. A time-course analyses of heat stress in rice seedlings demonstrated that the accumulation of H_2O_2 preceded the increase in GSH. This finding suggests that early augmentation of H_2O_2 levels during heat shock treatments acts as a signal to modulate GSH biosynthesis and to protect rice plants from Cd-induced damage. Xu et al. (2010) reported the H_2O_2-induced up-regulation of AsA and GSH metabolism-induced Al tolerance in wheat seedlings. Al stress resulted in higher $O_2^{\cdot-}$ and H_2O_2 contents, greater MDA levels, programmed cell death, and inhibited root growth in both rice genotypes. The activities of CAT, POD, SOD, GPX, GR, MDHAR, and DHAR and the levels of GSH and AsA increased in response to Al stress. However, H_2O_2-primed Al-stressed seedlings showed higher activities of GPX, CAT, POD, MDHAR, DHAR, and GR, and higher AsA and GSH contents as well as a more favorable redox state than seedlings subjected to Al-stress only. Notably, a large increase in ROS detoxifying enzyme activities was observed in the Al-susceptible genotype as compared with the resistant genotype. H_2O_2 pre-treatment increased the tolerance of plants to Al-induced oxidative stress by increasing level of GSH and AsA and the activities of enzymes involved in their metabolism. Bai et al. (2011) studied the effects of H_2O_2 pretreatment on Cd tolerance and translocation by utilizing two rice genotypes (N07-6 and N07-63) with contrasting Cd tolerance. Cd stress

(50 μmol/L) led a sharp decline in seedling growth and increased production of MDA, GSH, NPT, and phytochelatins (PCs), as well as GST activity. H_2O_2 pre-treatment further improved Cd stress tolerance by increasing the levels of NPT, GSH, and PCs, and the activity of GST in roots. The increase was greater in N07-63 as compared with the N07-6.

Chou et al. (2012) investigated the involvement of H_2O_2 in heat-shock induced Cd tolerance, in relation to the activities of ROS detoxifying enzymes (APX and GR) in rice plants. Heat-shock treatments increased the content of H_2O_2 before increases in the activities of APX and GR were observed in rice leaves. Importantly, heat-shock induced *OsAPX2* gene expression was associated with heat shock induced increases in APX activity. Upon imposition of Cd stress the H_2O_2 content and the activities of APX and GR increased, but the increase was less than that observed in seedlings subjected to Cd-stress without heat pre-treatment. The authors concluded that H_2O_2 is involved in the regulation of heat shock and Cd-induced increases in the activities of GR and APX in rice leaves, and thus cross-tolerance in rice plants. Yildiz et al. (2013) studied the ameliorating effects of H_2O_2 on chromium (Cr) toxicity in canola (*B. napus* L.) plants in relation to thiol content, lipid peroxidation, antioxidant enzyme activities and the growth and chlorophyll content, as well as the levels of a metallothionein protein (BnMP1). Cr stress (50 μM) significantly reduced plant growth, which was accompanied by increased lipid peroxidation and a decrease in the chlorophyll content of the leaves. H_2O_2 pre-treatment, enhanced plant growth reduced the MDA levels and promoted higher levels of pigments. Additionally, the accumulation of Cr was higher in the aerial parts of the H_2O_2-pretreated seedlings. Increased thiol levels were observed under Cr stress and were further enhanced by H_2O_2 pre-treatment. POD and SOD activities increased in response to Cr stress, whereas the activities of CAT and APX decreased. H_2O_2 pre-treatment caused an increase in the activities of APX and POD in response to Cr stress, but CAT and SOD activities remained unchanged. *BnMP1* expression analysis showed enhanced expression after 1 day of treatment, and then a decrease after 7 days exposure to Cr. In contrast, after 7 days exposure to Cr, H_2O_2-pre-treated seedlings showed a less decrease in *BnMP1* expression as compared with the seedlings subjected to Cr stress only. These findings indicate that H_2O_2 may act as a signal that triggers defense mechanisms that in turn protect plants from ROS-induced damage caused by exposure to Cr. In addition, the roles of exogenous H_2O_2 on plant growth, water status, mineral ion content, proline content, and total sugar and soluble protein contents were evaluated in maize leaves exposed to copper (Cu) stress (0.5 mM Cu) by Guzel and Terzi (2013). Cu stress resulted in a decrease in leaf water potential, ionic concentration (Na^+, K^+, Ca^+, Mg^{2+}) and protein levels, but increases in the content of proline content and total soluble sugars, when compared with controls. Importantly H_2O_2-pre-treated seedlings showed an increase in growth, water content, mineral concentration, proline content, total soluble sugar and soluble protein contents when compared with control plants. A greater increase was also observed in the proline and total sugar contents of the Cu+H_2O_2 group of plants compared with the Cu stress alone group. These findings suggest that exogenous H_2O_2 can increase dry matter production and mineral ion distribution in maize seedlings. Additionally, osmotic regulation might be involved in the alleviation of Cu toxicity of maize leaves caused by pre-treatment of H_2O_2.

Exogenous H_2O_2 and Multiple Stress Tolerance

The possible involvement of H_2O_2 in heat-induced cross-adaptation to salinity, drought, chilling and heat stress was studied by Gong et al. (2001) in two cultivars of maize differing in their stress tolerance. A heat-shock pre-treatment (42°C, 4 h) following 4-h recovery at a temperature of 28°C considerably enhanced the survival of seedlings, reduced the leakage of electrolyte from the roots and the loss of coleoptile vitality of seedlings after stress was imposed. Importantly, the heat-shock pre-treatment produced an H_2O_2 peak in the maize seedlings. The accumulation of H_2O_2 lead to the generation of cross-tolerance, indicating that an early short-lived increase in endogenous H_2O_2 is essential for the induction of cross-adaptation, by triggering increased expression of genes encoding ROS detoxification and therefore increasing the activities of antioxidant enzymes. Hence H_2O_2 could have a signaling role in inducing cross-tolerance in maize seedlings.

H_2O_2-Induced Abiotic Stress Tolerance and a Possible Biochemical and Molecular Basis

Recent studies have shown that H_2O_2 originating in the same cell can induce two dissimilar kinds of responses: one that depends on the subcellular sites of H_2O_2 production and the other integrating H_2O_2 signals independently of the subcellular site of production. Several studies have shown that H_2O_2 is a part of the retrogate signaling mechanism, from mitochondria or chloroplasts that activate the expression of nuclear-encoded stress-responsive genes. The aforementioned evidence clearly demonstrates that H_2O_2 priming modulates abiotic stress tolerance in plants by modulating ROS and MG detoxification and scavenging (Gong et al., 2001; Uchida et al., 2002; Yu et al., 2003; Xu et al., 2008, 2010; Hu et al., 2009; Jing et al., 2009; Gondim et al., 2010, 2012; Liu et al., 2010a; Wang et al., 2010a; Li et al., 2011; Bhattacharjee, 2012; Chou et al., 2012; Zhang et al., 2012; Hossain and Fujita, 2013; Ashraf et al., 2014; Sathiyaraj et al., 2014; Wang et al., 2014a), by enhancing the expression of heat shock proteins (Uchida et al., 2002; Wahid et al., 2007), by enhancing GSH and AsA biosynthesis (Yu et al., 2003; Hung et al., 2007; Chao et al., 2009; Xu et al., 2010; Bai et al., 2011; Wang et al., 2014a), by enhancing proline biosynthesis (Uchida et al., 2002; Guzel and Terzi, 2013; İşeri et al., 2013; Ashfaque et al., 2014; Sathiyaraj et al., 2014; Terzi et al., 2014), by enhancing photosynthesis (Wahid et al., 2007; Ishibashi et al., 2011; Moussa and Mohamed, 2011; Liao et al., 2012; Gondim et al., 2013), by enhancing ABA biosynthesis (Abass and Mohamed, 2011; Terzi et al., 2014) and by regulating multiple stress responsive pathways and genes. Based on the above findings we propose a hypothetical model that summarizes the possible mode of action

of H_2O_2 in planta (**Figure 2**). It is speculated that H_2O_2 priming induces a mild oxidative stress by disruption of cellular ROS homeostasis, from which develops a ROS-dependent signaling network and induces the accumulation of latent defense proteins such as ROS-scavenging enzymes and transcription factors (TFs), among others, resulting in a primed state and enhanced stress responses. Another notable effect of H_2O_2 is its capacity to induce the expression of TFs and genes responsible for osmolyte synthesis, e.g., proline and betaine, and activate phosphorylation cascades using mitogen-activated protein kinases (MAPKs). In the following section we will critically discuss the perception of extracellular and intracellular H_2O_2 by plants and how this signal transduces and modulates the abiotic stress tolerance of plants.

H_2O_2 as a Signaling Molecule Involved in Stress Tolerance

Sensing of H_2O_2

H_2O_2 can act as a signaling molecule via chemical reactions with targeted amino acids that can lead to peptide/protein modifications. It is now well established that GSH and protein cysteine (Cys) residues are particularly well suited for reactions with oxidants such as H_2O_2 (D'Autréaux and Toledano, 2007; Munné-Bosch et al., 2013). Cys residues in proteins are one of the most sensitive targets for ROS-mediated posttranslational modifications, and have become the focus of many ROS signaling studies. The electron-rich sulfur atom makes Cys residues the major sites of oxidative modifications within proteins (Akter et al., 2015). It is a well-recognized concept that profiling of ROS/RNS-modified proteins containing Cys can be used to help identify key redox sensors involved in signal transduction pathways (Couturier et al., 2013). Heat stress transcription factors (Hsfs) may also function as ROS-dependent redox-sensors. Hsf proteins contain a DNA binding domain, control the transcription of heat stress associated genes (Baniwal et al., 2004) and are distributed both in the cytosol (mainly in the inactive form) and in the nucleus. Once Hsfs enter the nucleus, they bind to the heat shock elements of the promoters of ROS-sensitive genes, such as the gene encoding APX. There is evidence that certain Hsfs directly sense ROS and control gene expression during oxidative stress (Miller and Mittler, 2006). For example, the transcription of genes encoding the cytosolic peroxidases APX1 and APX2 can be regulated by Hsfs, which in turn can be modulated by ROS (Mazars et al., 2009). Increased expression of Hsfs caused by exogenous H_2O_2 was also found to modulate salinity tolerance (Uchida et al., 2002; Wahid et al., 2007). Transgenic *B. napus* plants over-expressing the *Arabidopsis* HEAT SHOCK TRANSCRIPTION FACTORA1b showed enhanced resistance to drought (Bechtold et al., 2013).

Signal Transduction, Stress Tolerance and Stress Cross-Tolerance

H_2O_2 signaling appears to be integrated with many different signaling networks in plant cells, like Ca^{2+}-signaling, protein kinase networks, cellular metabolic networks etc. In some cases, H_2O_2 and ROS accumulation was found to precede the activation of signaling, whereas in other cases H_2O_2 accumulation was found to be a consequence of signaling. Signal transduction components including protein kinases, such as calcium-dependent protein kinases (CDPKs) and mitogen-activated protein (MAP) kinases have been implicated in stress tolerance as well as cross-tolerance between biotic and abiotic stress responses (Wurzinger et al., 2011). MAPK cascades are important pathways in abiotic stress responses and enable extracellular stimuli to be transduced into intracellular changes (Zhou et al., 2014). A number of cellular stimuli that induce ROS (H_2O_2) production can also activate MAPK pathways in multiple cell types (Torres and Forman, 2003; McCubrey et al., 2006). H_2O_2- and ROS-responsive MAPKKK, MAPK1,

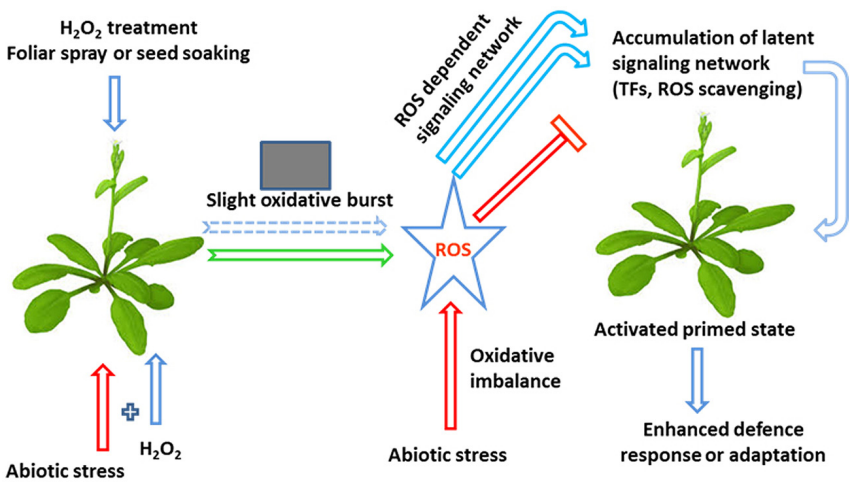

FIGURE 2 | A hypothetical model of the influence of H_2O_2 on plant defense mechanisms associated with abiotic stresses. H_2O_2 treatment is capable of inducing abiotic stress tolerance through the development of a small oxidative burst. This burst subsequently activates a ROS-dependent signaling network, thereby enhancing the accumulation of latent defense proteins, such as ROS-scavenging enzymes and transcription factors (TFs), resulting in a primed state and an enhanced stress response (modified from Borges et al., 2014).

MAPK4, and MAPK6 usually remain highly active under oxidative stress and redox regulation of environmental stress responses. Direct exposure of cells to exogenous H_2O_2 leads to activation of MAPK pathways (Dabrowski et al., 2000; Ruffels et al., 2004). The MEKK1 pathway, which was found to be highly active under oxidative stress and induced by unfavorable environmental conditions seems to be the activator of two MAPKKs (MKK1 and MKK2), that in turn activate other MAP kinases (Mittler et al., 2011). MEKK1 is thought to be needed for the activation of MAPK4 by H_2O_2 (Mittler et al., 2011). Similarly, MAPK12 was found to be up regulated in response to ABA and H_2O_2 application (Mittler et al., 2011). MKP2 is a key regulator of the MPK3 and MPK6 networks that are involved in controlling both abiotic and biotic stress responses (Jammes et al., 2009; Mittler et al., 2011). Zhou et al. (2012) reported the regulatory role of H_2O_2 in cold acclimation-induced chilling tolerance in tomato. Cold acclimation induces a modest increase in H_2O_2, *RBOH1* gene expression and NADPH oxidase activity that modulates the expression and activity of ROS detoxifying enzymes and ensures stress cross-tolerance. Zhou et al. (2014) further proved the involvement of apoplastic H_2O_2 in modulating stress cross-tolerance. Tomato plants pre-treated with mild cold, paraquat (PQ), or drought pre-treatment modulated abiotic stress tolerance by increasing the endogenous level of H_2O_2 in the apoplast, which is well correlated with *RBOH1* transcription. An enhanced H_2O_2 level was found to modulate the expression of stress and defense-related genes, increase the activities of SOD, APX, CAT, and GR, maintain higher GSH/GSSG ratio and activate MPK1/2. Their findings support the involvement of H_2O_2 and MPK1/2 in cross-tolerance in plants. The exogenous application of H_2O_2 to *A. thaliana* can activate the MAPK cascades that regulate ROS production and detoxification (Pan et al., 2012). In some cases, MPK3/6 responses to cadmium (Cd) treatment are mediated by the H_2O_2-signaling pathway, where MPK3/6 is upregulated after an accumulation of H_2O_2 (Liu et al., 2010b). H_2O_2 may also be involved in the MAP kinase 8 (MPK8) pathway, since expression of *RBOHD* rapidly decreases via MPK8, resulting in negative regulation of H_2O_2 synthesis (Takahashi et al., 2011).

Ca^{2+} one of the most important second messenger in the sophisticated network of plant abiotic stress signaling (Dodd et al., 2010; Wei et al., 2014) and regulation of Ca^{2+} homeostasis is one of the main targets of H_2O_2 signaling (Petrov and Van Breusegem, 2012). Regulation of guard cells during stomatal opening is one of the most widely studied processes involving H_2O_2-mediated Ca^{2+} signaling and Ca^{2+} is a central signaling component in guard cell responses to stimuli like ABA, ROS and NO (Allen et al., 2000; Young et al., 2006). Ca^{2+} homeostasis also regulates antioxidative defenses in plants. An increase in the concentration of intracellular Ca^{2+} causes efficient detoxification of H_2O_2 and involves increased levels of detoxification enzymes, including Ca^{2+}-sensitive CAT3. Application of the Ca^{2+} channel blocker $LaCl_3$, Ca^{2+} chelator (EGTA) or the calmodulin (CaM) inhibitor (trifluroperazine) to germinating *Amaranthus* seeds, causes a significant reduction in the levels of H_2O_2-scavenging enzymes (Bhattacharjee, 2008), strongly supporting the role of Ca^{2+} as a regulator of H_2O_2 titer in plant tissues. A rapid increase in cellular Ca^{2+} is considered as one of the earliest responses associated with H_2O_2 signaling. Wu et al. (2010) showed that spermidine oxidase-derived H_2O_2 regulates pollen membrane hyper-polarization-activated Ca^{2+} channels in order to induce pollen tube growth. In contrast, cytoplasmic Ca^{2+} is able to trigger changes in H_2O_2 levels. It is also evident that H_2O_2 synthesis requires a continuous cytoplasmic influx of Ca^{2+}, which activates NADPH oxidases located at the plasma membrane (Lamb and Dixon, 1997). The Ca–CaM signaling pathway also regulates a number of different target proteins in signaling cascades, including MAP kinases. MAP kinase pathways in turn negatively regulate H_2O_2 synthesis by up-regulating the expression of *RbohD*. Thus, CDPKs are involved in tolerance to abiotic stresses (Wei et al., 2014). Treatment of tomato and wheat leaves with H_2O_2 increased the expression of CDPKs (Chico et al., 2002; Li et al., 2008). Costa et al. (2010) showed that CAT scavenging of H_2O_2 is regulated through a Ca^{2+}-dependent pathway in the peroxisomes of *Arabidopsis* guard cells. Recent evidence suggests that RBOH-dependent H_2O_2 production might be mediated by Ca^{2+} homeostasis in *Arabidopsis* (Suzuki et al., 2011). In this case, cytoplasmic Ca^{+2} was shown to bind to the EF-hands of the N-terminal region of RBOH and thus promote the activation of RBOH and the production H_2O_2 (Takeda et al., 2008). The Ca^{2+} channels and transporters activated by these stimuli form specific Ca^{2+} signatures and changes in these Ca^{2+} signatures are transmitted by protein sensors that preferentially bind Ca^{2+}. The binding of Ca^{2+} results in conformational changes in these protein sensors that modulate their activities or their ability to interact with other proteins, and activate the expression of downstream salt-responsive genes through a Ca^{2+} signaling cascade (Rentel and Knight, 2004; Dodd et al., 2010; Kudla et al., 2010; Batistic and Kudla, 2012).

WRKY and zinc finger TFs are both widely involved in the regulation of ROS-related defense genes. It was observed that the ZAT7 and ZAT12 zinc finger proteins of *Arabidopsis* are strongly up regulated by oxidative stress in *apx* knockout mutants in response to H_2O_2 and methyl viologen (MV) treatment (Rizhsky, 2004). ZAT10 has a dual role both as an inducer and as a repressor of ROS-responsive genes under salt, drought and osmotic stresses (Sakamoto et al., 2004; Mittler, 2006). ZAT6 positively regulates tolerance to drought, salt, and chilling stress, as well as resistance to bacterial infection, by modulating ROS levels and SA-related gene expression (Shi et al., 2014). Accumulation of NO under stressful conditions was found to initiate defense responses similar to those seen following H_2O_2 production (Wang et al., 2014b). NO and H_2O_2 are also involved in the stimulation of stomatal closure in *Arabidopsis* in response to ultraviolet-B exposure (He et al., 2005). Removal of H_2O_2 with antioxidants or inhibition of its synthesis by inhibiting NADPH oxidase activity prevents NO generation and stomatal closure. Recent evidence supports the idea that H_2O_2-induced synthesis of NO might be mediated by MPK6 in *Arabidopsis* (Wang et al., 2010b).

The links between H_2O_2 signaling and other signaling pathways are summarized in **Figure 3**. These demonstrate a complex interaction between H_2O_2 signaling, environmental and

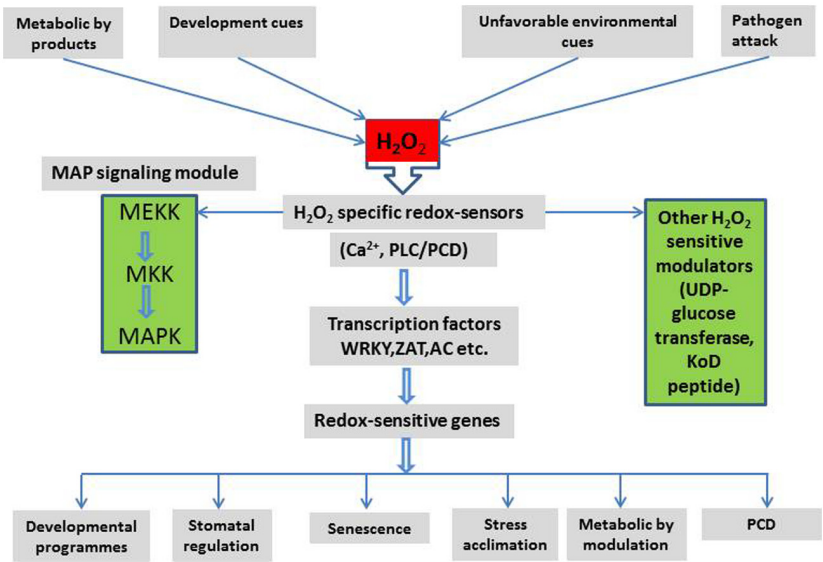

FIGURE 3 | A schematic representation of major signaling components and their interactions in the H_2O_2 signaling network and the possible outcome in plant cells (Detail in text).

metabolic cues, their down-stream effects and plant development. It appears that H_2O_2 may be involved, in one way or another, in every aspect of plant cell signaling, development, stress responses, and stress tolerance.

Gene Expression and H_2O_2-Priming and Abiotic Stress Tolerance

Adaptation, the important responses of plants to environmental stresses, is related to environmental stimulation, signal transduction, gene expression, and cellular metabolic adjustment (Pastori and Foyer, 2002). Plants, like other living organisms, sense increased levels of ROS and react with antioxidant mechanisms, finely coordinated, and expressed, to effectively protect the organism from oxidative stress. Modulation of gene expression by H_2O_2 has received much attention as H_2O_2 is generated in response to a variety of stress stimuli and it is likely to mediate crosstalk between different signaling pathways (Bowler and Fluhr, 2000). H_2O_2 modulates the expression of genes involved in ROS control, signal transduction, transcriptional regulation, and protein, carbohydrate, and lipid metabolism, demonstrating the complexity of the transcriptional responses to H_2O_2 (Li et al., 2011). A large number of genes involved in stress perception, signal transduction, transcription, defense, and general metabolism have been identified, revealing a highly dynamic and redundant network of genes associated with enzymes involved in ROS-production and ROS-scavenging. In most cases, H_2O_2 seems to be positively used by plants to activate multiple stress-responsive genes that help cope with environmental changes. Genes encoding antioxidant enzymes are central players in this network and their function has profound effects in controlling excessive ROS accumulation and cellular redox balance. In contrast, ROS can also negatively regulate the expression of genes encoding antioxidant enzymes, providing a feedback mechanism that can in turn regulate ROS levels, which is a critical component in the modulation of signaling networks (Mylona and Polidoros, 2010). A number of studies have shown that manipulation of plant antioxidant defenses results in cross-tolerance to subsequent exposure of plants to conditions that cause oxidative stress (Neill et al., 2002). Short-term exposure of CAT-deficient mutants to H_2O_2 can trigger an increase in tolerance to subsequent severe oxidative stress, indicating that cross-tolerance is mediated by H_2O_2 (Vanderauwera et al., 2005). The pivotal role of peroxisomal CAT in decomposing photorespiratory H_2O_2 and modulating the signaling role of H_2O_2 was recently shown in *Arabidopsis* CAT2-deficient plants (Vandenabeele et al., 2004; Queval et al., 2007). Specifically, photorespiration-generated H_2O_2 modulates nuclear transcriptional programs influencing the expression of cytosolic, chloroplastic, and mitochondrial genes, providing additional evidence for the importance of interorganelle communication as part of a plant's defense response.

Polidoros and Scandalios (1999) showed that high concentrations of H_2O_2 rapidly induced *CAT* and *GST1* expression, indicating that oxidative stress directly induces antioxidant responses. They also showed that H_2O_2 induced expression of a *GST* gene in the leaves of maize seedlings (Polidoros et al., 2005). GST comprises of a family of nuclear-encoded enzymes involved in cellular detoxification processes following various abiotic stresses, including exposure to xenobiotics and metals (Mylona et al., 2007). Recently, Wang et al. (2013) showed that H_2O_2 is involved in the regulation of rice (*O. sativa* L.) tolerance to salt stress where exogenous H_2O_2 significantly enhances the activities of APX, CAT, POD, SOD, and G6PDH in a concentration-dependent manner in rice roots. *GPX1* promoter analysis showed that salt induction is mediated

via ROS (predominantly H_2O_2) in an intracellular process, whereas induction by exogenous H_2O_2 involves a different signaling pathway that involves NADPH oxidase. Surprisingly, the promoter of *GPX1* did not respond to exogenous ABA, although *GPX1* transcripts increased in response to ABA in citrus (*Citrus sinensis*) plants. In *Arabidopsis*, it was demonstrated that GPX relays the H_2O_2 signal to other signaling molecules, such as ABA (Dietz, 2008). Accumulation of H_2O_2 opens Ca^{2+} channels, and induces the generation of Ca^{2+}/CaM complexes and the activation of MAPK cascades. TF encoding genes, such as *ZAT10*, *ZAT12,* and *ABI4*, are induced in response to various abiotic and biotic stresses. Transcriptome analysis has shown that some nuclear-encoded TF encoding genes can be induced specifically by the accumulation of a particular ROS (H_2O_2), while others are induced by all ROS (Scarpeci et al., 2008). Also a number of TFs have been shown to modulate other antioxidant gene responses. H_2O_2 also functions as a major stress signal molecule in plants and the expression of at least 1–2% of *Arabidopsis* genes is now known to be H_2O_2-dependent (Desikan et al., 2001). These gene expression studies clearly show that increased cellular H_2O_2 levels have a considerable impact on the transcriptome of all plant species, by changing the expression of 100s of genes. These genes are generally involved in cell wall protection, desiccation tolerance, production of ROS scavenging enzymes and DNA damage repair.

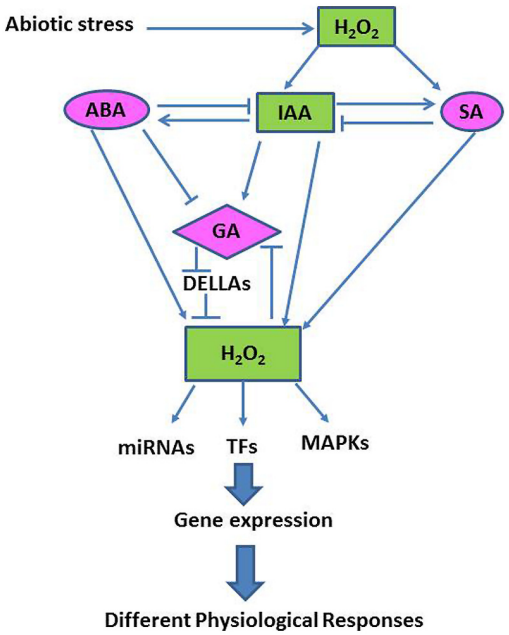

FIGURE 4 | A simplified schematic model showing interactions between the H_2O_2-transduced network and different hormonal signaling pathways in the plant cell (Adapted from Habibi, 2014).

Ultrastructure Protection by Exogenous H_2O_2 Pretreatment and Abiotic Stress Tolerance

Disorganization and/or damage to chloroplast and mitochondrial ultrastructures have been reported in plants in response to excessive accumulation of ROS induced by abiotic stresses (Yamane et al., 2003; Xu et al., 2008; Liu et al., 2010a; Huang et al., 2015). The alteration of chloroplast or mitochondrial ultrastructures was correlated with higher ROS (H_2O_2) and MDA accumulation, as well as lower antioxidant enzyme activities under stressful conditions (Xu et al., 2008; Liu et al., 2010a; Huang et al., 2015). H_2O_2 pre-treatment induced stress tolerance was associated with higher antioxidant enzyme activities and lower levels of endogenous ROS accumulation (Liu et al., 2010a; Huang et al., 2015). These findings indicate that effective ROS metabolism is essential for maintaining the structural integrity of cellular organelles and keeping them fully functional in plants under abiotic stresses. H_2O_2-primed plants can overcome the adverse stress conditions through better ROS metabolism, as well as through the protection of cellular organelles indirectly.

H_2O_2, Hormones and Abiotic Stress Tolerance

Although the precise mechanisms associated with abiotic stress tolerance remain to be fully elucidated, recent studies have found that many abiotic stress responses are coordinated by complex signaling networks, involving both phytohormones and ROS, especially H_2O_2 (Bartoli et al., 2013; **Figure 4**). Here we discuss examples demonstrating that major plant hormones interact with H_2O_2 and other signaling pathways in modulating plant growth and abiotic stress tolerance.

Abscisic acid is not only an important plant hormone that regulates plant growth and development but also a global regulator that mediates abiotic stress responses. Abiotic stresses can induce an accumulation of ABA (Jia et al., 2002), moreover ABA can induce the production of ROS in plant cells and H_2O_2 production in guard cells plays an important role in ABA-mediated stomatal closure (Kwak et al., 2003), while NO production acts downstream of H_2O_2 in ABA-induced stomatal closure (Bright et al., 2006). In addition to its function in stomatal closure, ABA also plays crucial role in ROS-mediated stress tolerance. In maize, water stress or ABA treatment influences the accumulation of ROS and increases antioxidant enzyme activities (Jiang and Zhang, 2002), whereas ROS scavengers completely block the ABA-mediated increases in antioxidant activities (Zhang et al., 2006). The expression of NADPH oxidase encoding genes and H_2O_2 accumulation in *Arabidopsis* is induced immediately after exposure to salt stress or ABA treatment. H_2O_2 accumulation can be suppressed by an inhibitor of NADPH oxidases (DPI) and DPI-treated plants show reduced salt stress tolerance (Zhang et al., 2001; Kwak et al., 2003; Leshem et al., 2007). During ABA-mediated stress responses, MAPK cascades may act both upstream and downstream of ROS production (Zhang et al., 2006; Zhou et al., 2014). MPK9 and MPK12 were identified as downstream factors that integrate ABA-ROS signaling, leading to anion channel activation (Jammes et al., 2009). It was reported that H_2O_2 is an essential signal in mediating stomatal closure induced by ABA via activation of Ca-permeable channels in the plasma membrane under a variety of abiotic stresses, such as drought and salinity (Pei et al., 2000; Neill et al., 2002; Kim et al., 2010). In addition, cytokinin-deficient mutants have been found to be tolerant to drought and

salinity and have ABA hypersensitivity and reduced levels of ABA (Nishiyama et al., 2011).

Jasmonic acid (JA) and methyl jasmonate (MeJA, a biologically active derivative of JA) are well-established signal molecules involved in a plant's defense responses and are effective inducers of H_2O_2 accumulation in plant cell cultures (Wang and Wu, 2004, 2013). Pre-treatment of *Arabidopsis* and tobacco plants with JA abrogated O_3-induced H_2O_2 accumulation, SA production, and defense gene activation (Overmyer et al., 2000; Rao et al., 2000, 2002). MeJA might also play an important role in signal transduction in grape cells, regulating the levels of NO and H_2O_2 and enhancing the activities of enzymes involved in phytoalexin synthesis (Wang et al., 2012). Like ABA, MeJA can induce stomatal closure mediated by ROS-dependent signaling pathways (Suhita et al., 2004). JA can also interact with ABA, ethylene (ET) and SA in oxidative stress triggered by water deficiency (Brossa et al., 2011). Increased JA accumulation under drought was found to increase the transcript abundance and activities of APX, GR, MDHAR, DHAR, and L-galactono-1,4-lactone dehydrogenase (GalLDH), as well as enhancing the contents of AsA and GSH (Shan and Liang, 2010). During water stress, transient accumulation of JA is needed for ABA increases in citrus roots (de Ollas et al., 2013).

Salicylic acid (SA) plays an important role in plant responses to various types of environmental stresses, including drought, low temperature and high salinity (Kang et al., 2014; Miura and Tada, 2014). Under stress, the levels of endogenous SA and H_2O_2 increase in plant cells. However, the levels H_2O_2 degrading enzymes, such as CAT and APX, can be suppressed by SA treatment (Yuan and Lin, 2008). Previous studies have shown that H_2O_2 alone is not sufficient to trigger antioxidant defense responses and cell death in cell cultures (Chen et al., 1993). However, a SA-deficient transgenic *Arabidopsis* line overexpressing salicylate hydroxylase (*NahG*) gene exposed to O_3 accumulated more H_2O_2 compared with non-transgenic plants, yet failed to efficiently induce defense genes, such as *GST1* and *GPX*, and perturb the redox state of glutathione (Rao et al., 2002). This indicates that H_2O_2 alone is not sufficient to trigger defense responses, and SA is required to maintain the cellular redox state and potentiate defense responses in O_3-exposed plants (Rao and Davis, 1999). Pre-treatment with SA causes ROS accumulation (Mittler, 2002; Harfouche et al., 2008). ROS production mediated by salicylhydroxamic acid (SHAM)-sensitive guaiacol peroxidases was induced by SA in guard cells, which may be independent of the ABA pathway (Mori et al., 2001; Khokon et al., 2011). Other enzymes inhibited by SA, such as APX, CAT, and carbonic anhydrase, are also involved in scavenging ROS (Chen et al., 1993; Conrath et al., 1995; Durner and Klessig, 1995; Slaymaker et al., 2002). SA and ROS, mainly H_2O_2, have been proposed to form a self-amplifying feedback loop in response to abiotic and biotic stresses (Vlot et al., 2009).

Auxins have been shown to play an important role in stress-related hormonal networks. They can indirectly modulate ROS homeostasis by affecting the stability of DELLA proteins (Fu and Harberd, 2003; Weiss and Ori, 2007; Paponov et al., 2008) or directly by inducing ROS detoxification enzymes, such as GSTs and quinine reductases (Laskowski et al., 2002). Analysis of the TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/AUXIN SIGNALLING F-BOX PROTEINS (AFB) auxin receptors shows that these genes are involved in tolerance to oxidative stress by regulating H_2O_2, antioxidant enzymes, antioxidant levels and the chlorophyll content of plants (Iglesias et al., 2010). In addition, auxin-induced H_2O_2 production acts as a signal for the stomatal opening response (Song et al., 2006). H_2O_2 is also involved in auxin-induced root geotropism (Joo et al., 2001). Any exogenous or endogenous stimulus that perturbs cellular redox balance can activate auxin homeostasis because NADPH oxidase-dependent ROS production influences polar auxin transport (Joo et al., 2001, 2005).

Gibberellic acids (GAs) mediate growth in response to environmental signals by relieving the constraints on gene expression imposed by a family of growth-repressing regulators, the DELLA proteins (Peng et al., 1999; Harberd et al., 2009). Moreover, the *Arabidopsis* GA-deficient *ga1-3* mutants, that have higher levels of DELLA proteins, are much less susceptible to ROS-dependent cell death (Achard et al., 2008). Stress (drought or high salt) conditions enhance ABA/GA ratios favoring DELLA protein accumulation and lower ROS levels (Finkelstein et al., 2008; Considine and Foyer, 2014). ABA can decrease ROS production in rice seeds, leading to a repression of ascorbate and GA accumulation (Ye et al., 2012). Recently, it was reported that ROS are involved in GA/ABA signaling in barley aleurone cells (Ishibashi et al., 2012). In addition, GA-stimulated *Arabidopsis* gene *GASA14* regulates leaf expansion and abiotic stress tolerance by modulating ROS accumulation (Sun et al., 2013). The overexpression of *GASA4* suppressed ROS accumulation in transgenic plants and enhanced their tolerance to NO (Rubinovich and Weiss, 2010).

Ethylene can induce ROS generation and H_2O_2 can stimulate the expression of ET biosynthesis and responsive genes (Vandenabeele et al., 2003). The PCD pathways occurring in leaf abscission depends on NADPH oxidase-dependent H_2O_2 generation triggered by ET (Sakamoto et al., 2008). Moreover, stomatal closure also requires the integrated cooperation of H_2O_2 and ET. In *Arabidopsis*, the ETHYLENE RESPONSE FACTOR1 (ERF1) is greatly induced by high salinity and drought stress (Cheng et al., 2013). Induction of the salt stress response in *Arabidopsis* required both JA and ET signaling, but was inhibited by ABA. Transgenic plants overexpressing ERF1 showed higher salt and drought tolerance and less water loss due to transpiration (Cheng et al., 2013).

From the above discussion, it is evident that ROS production and associated redox processing are an integral part of hormone regulation and functioning in the control of plant abiotic stress responses and tolerance. Further understanding of H_2O_2-hormone-antioxidant interactions will help to facilitate the development of plants tolerant to various abiotic stresses.

Conclusion and Future Perspectives

H_2O_2 priming represents a fruitful area of future research, which should help plant scientists explore the molecular mechanisms associated with abiotic stress tolerance and promote

a more environmental friendly sustainable agriculture. Plants have mechanisms, particularly under environmental stress, to utilize ROS, especially H_2O_2, for signaling purposes that confer acclamatory stress tolerance through the modulation of osmotic adjustment, ROS detoxification, photosynthetic C fixation and hormonal regulation. A large number of studies have suggested that pre-treatment of seeds or seedlings with H_2O_2, or the combined application of H_2O_2 and abiotic stress, induces an inductive pulse that helps to protect plants under abiotic stresses by restoration of redox-homeostasis and mitigation of oxidative damage to membranes, proteins and lipids and by modulating stress signaling pathways (**Figures 2–4**), however, the mechanisms for this are not well established. Many researchers have suggested a central role for H_2O_2 in intracellular and systemic signaling routes that increase tolerance and acclimation to abiotic stresses bur how H_2O_2 is sensed by plant cells is still a mystery, and numerous factors are still being considered with respect to their roles in H_2O_2-induced signal transduction and development of responses to oxidative stress. Recent findings have shown that effective ROS signaling may require an increased flux of antioxidants, notably those that are thiol-dependent. With respect to signal transduction, ROS can interact with other signaling pathways, such as the activation of NADPH oxidase dependent or monomeric G protein; lipid-derived signals; induction of MAPK; redox sensitive TFs; regulation of Ca^{2+}; and plant hormone signal transduction. An understanding of the H_2O_2 physiology of plants, particularly H_2O_2 sensing and the identification of the components of H_2O_2 signaling network and H_2O_2 cross-talk with other growth factors, is necessary and is of great importance if we are to improve the performance of crop plants growing under conditions that cause abiotic stress.

Identification of the genetic network and of the downstream reactions modulated by H_2O_2 originating from specific organelles remains a future challenge. Additionally, one of challenges in H_2O_2 research is to identify specific H_2O_2 receptors and to establish how the cell is able to decode endogenous H_2O_2 signals and discriminate between different stimuli giving rise to a very specific defense response. Utilization of genetically engineered plants that suppress H_2O_2 generation or increase H_2O_2 production and the isolation of H_2O_2-signaling mutants will be invaluable in elucidating further the biological roles of H_2O_2 in specific cells and in the response to various abiotic stressors that cause oxidative damage. Further proteomic, metabolomic and transcriptomic studies will provide further insights into the responses of cells to H_2O_2. Future research is likely to find new signaling roles for H_2O_2 in regulating ROS scavenging systems with respect to modulating abiotic stress tolerance. A better understanding of H_2O_2 and its role in regulating ROS scavenging systems will be valuable in helping produce crop plants with greater levels of tolerance to oxidative stress using traditional plant breeding or biotechnological approaches. Understanding the subtle and sensitive mechanisms plants use to fine-tune H_2O_2 titer and the associated signaling cascades may hold the key to improving agriculture in the future.

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Hydrogen Peroxide Alleviates Nickel-Inhibited Photosynthetic Responses through Increase in Use-Efficiency of Nitrogen and Sulfur, and Glutathione Production in Mustard

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The response of two mustard (*Brassica juncea* L.) cultivars differing in photosynthetic capacity to different concentrations of hydrogen peroxide (H_2O_2) or nickel (Ni) was evaluated. Further, the effect of H_2O_2 on photosynthetic responses of the mustard cultivars grown with or without Ni stress was studied. Application of 50 μM H_2O_2 increased photosynthesis and growth more prominently in high photosynthetic capacity cultivar (Varuna) than low photosynthetic capacity cultivar (RH30) grown without Ni stress. The H_2O_2 application also resulted in alleviation of photosynthetic inhibition induced by 200 mg Ni kg^{-1} soil through increased photosynthetic nitrogen-use efficiency (NUE), sulfur-use efficiency (SUE), and glutathione (GSH) reduced production together with decreased lipid peroxidation and electrolyte leakage in both the cultivars. However, the effect of H_2O_2 was more pronounced in Varuna than RH30. The greater increase in photosynthetic-NUE and SUE and GSH production with H_2O_2 in Varuna resulted from higher increase in activity of nitrogen (N) and sulfur (S) assimilation enzymes, nitrate reductase and ATP-sulfurylase, respectively resulting in enhanced N and S assimilation. The increased N and S content contributed to the higher activity of ribulose-1,5-bisphosphate carboxylase under Ni stress. Application of H_2O_2 also regulated PS II activity and stomatal movement under Ni stress for maintaining higher photosynthetic potential in Varuna. Thus, H_2O_2 may be considered as a potential signaling molecule for augmenting photosynthetic potential of mustard plants under optimal and Ni stress conditions. It alleviates Ni stress through the regulation of stomatal and non-stomotal limitations, and photosynthetic-NUE and -SUE and GSH production.

Keywords: glutathione, hydrogen peroxide, nickel, photosynthesis, photosynthetic-NUE, photosynthetic-SUE

INTRODUCTION

Nickel (Ni) is an essential metallic micronutrient with important roles in plant metabolism. However, excess Ni added to soil due to the increased anthropogenic activities becomes toxic and affects growth and development of plants adversely (Chen et al., 2009; Anjum et al., 2014). The inhibition of metabolic processes in plants by high Ni is associated with the production of excess

reactive oxygen species (ROS) causing injury to DNA, oxidation of proteins and lipids and damage to photosynthetic apparatus (Gajewska et al., 2006; Asgher et al., 2015). It has been shown that hydrogen peroxide (H_2O_2) plays dual roles in plants under optimal and stressful environments, as it initiates programmed cell death at high concentration and promotes physiological processes at lower concentration (Dat et al., 2000). It acts as a signaling molecule for acclimation and tolerance to various stress factors such as salt (Li et al., 2011), heat (Bhattacharjee, 2013), cold (İşeri et al., 2013), osmotic (Pongprayoon et al., 2013) chilling (Wang et al., 2010), and cadmium (Zelinová et al., 2013). Thus, studies to establish the role of H_2O_2 as a secondary messenger molecule (Neill et al., 2002; Hung et al., 2005) and modulator of abiotic stress responses (Uchida et al., 2002; de Azevedo Neto et al., 2005; Wang et al., 2010; Li et al., 2011; Pongprayoon et al., 2013) in plants under normal or stress conditions have gained attention in recent years. Recently, Ashfaque et al. (2014) have shown that exogenous application of H_2O_2 promoted photosynthesis and growth of wheat plants grown under salt stress.

Plants up-regulate production of enzymatic and non-enzymatic antioxidants under stress conditions that cooperatively manage to remove/neutralize or scavenge ROS (Noctor et al., 2012) and maintain high rates of photosynthesis (Foyer and Shigeoka, 2011). The regulation of glutathione (GSH) reduced production through modulating nitrogen (N) and sulfur (S) may help in the alleviation of Ni stress-accrued photosynthetic anomalies in crop plants as the availability of N and S influences GSH production and photosynthesis (Iqbal et al., 2011). Therefore, the increased photosynthetic nitrogen use efficiency (NUE) and photosynthetic sulfur use efficiency (SUE) are expected to alleviate Ni-inhibited photosynthetic responses by maintaining the GSH production. The reduction in photosynthetic-NUE and photosynthetic-SUE has been shown to reduce photosynthesis (Iqbal et al., 2012; Khan and Khan, 2014). There are reports that show increase in photosynthesis with H_2O_2 (Ashfaque et al., 2014), but detailed studies on the physiological processes and biochemical mechanisms to alleviate Ni stress are not available. In the present study, we analyzed the effects of exogenous H_2O_2 on N and S-assimilation and GSH production to examine whether these processes result in improved photosynthetic responses in two cultivars of mustard that differ in photosynthetic capacity under Ni stress.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Seeds of mustard (*Brassica juncea* L. Czern and Coss.) cultivars, Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity; Mobin and Khan, 2007) were surface sterilized with 0.01 g L^{-1} $HgCl_2$ solution followed by repeated washings and sown in 23-cm diameter pots filled with reconstituted soil [peat and compost, 4:1 (v/v); mixed with sand, 3:1 (v/v)]. After seedlings establishment, three healthy plants of nearly equal size in each pot were maintained. The pots were kept in the naturally illuminated net house of the Botany Department, Aligarh Muslim University, Aligarh (India) with day/night temperatures at 24°C/18°C (±3°C), relative humidity of 68 ± 5%; and the pots were watered daily with 250 mL deionized water. Experiments were conducted independently to study the effect of 0, 50, 100, and 200 mg Ni kg^{-1} soil or 0, 25, 50, and 100 μM H_2O_2 applied basally to plants 15 days after seed germination on lipid peroxidation, GSH content, and photosynthesis and plant dry mass of Varuna and RH30. As 50 μM H_2O_2 maximally increased photosynthesis and 200 mg Ni kg^{-1} soil was the most toxic, these concentrations were taken for further study. In another experiment, Varuna and RH30 plants were grown in the similar conditions and were treated with 0 (control) or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Nickel chloride ($NiCl_2$) was used for Ni treatment. The experiment followed a factorial randomized complete block design and the number of replicates for each treatment was four ($n = 4$). Sampling of plants was done at 30 days after sowing (DAS) to record different parameters.

Determination of Lipid Peroxidation and Electrolyte Leakage

Lipid peroxidation in leaves was determined by estimating the content of thiobarbituric acid reactive substances (TBARSs) as described by Dhindsa et al. (1981). Fresh leaf tissues (500 mg) were ground in 0.25% 2-thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using mortar and pestle. After heating at 95°C for 30 min, the mixture was rapidly cooled on ice bath and centrifuged at 10,000 *g* for 10 min. To 1 mL of the supernatant 4 mL 20% TCA containing 0.5% TBA was added. The absorbance of the supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance of the same at 600 nm. The content of TBARS was calculated using the extinction coefficient (155 mM^{-1} cm^{-1}).

For measuring electrolyte leakage, samples were thoroughly washed with sterile water, weighed and then kept in closed vials with 10 mL of deionized water and were incubated at 25°C for 6 h using shaker and electrical conductivity (EC) was determined (C_1). Samples were then again kept at 90°C for 2 h and EC was recorded after attaining equilibrium at 25°C (C_2).

Assay of Antioxidant Enzymes

Fresh leaf tissue (200 mg) was homogenized with an extraction buffer containing 0.05% (v/v) Triton X-100 and 1% (w/v) polyvinylpyrrolidone (PVP) in potassium phosphate buffer (100 mM, pH 7.0) using chilled mortar and pestle. The homogenate was centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatant obtained after centrifugation was used for the assay of glutathione reductase (GR; EC 1.6.4.2) enzyme. For the assay of ascorbate peroxidase (APX; EC 1.11.1.11), extraction buffer was supplemented with 2 mM ascorbate.

Ascorbate peroxidase activity was determined by the method of Nakano and Asada (1981). Ascorbate peroxidase activity was determined by the decrease in the absorbance of ascorbate at 290 nm due to its enzymatic breakdown. The volume of 1 ml of 50 mM K-phosphate buffer (pH 7.2) contained

0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA, and 0.1 ml enzyme extract. The reaction was allowed to run for 5 min at 25°C. Ascorbate peroxidase activity was calculated by using the extinction coefficient 2.8 mM^{-1} cm^{-1}. One Unit of enzyme activity is defined as the amount necessary to decompose 1 μmol of substrate consumed per min at 25°C.

Glutathione reductase activity was determined by the method of Foyer and Halliwell (1976). Glutathione reductase activity was determined by monitoring the glutathione dependent oxidation of NADPH at 340 nm. The assay mixture (3 mL) contained phosphate buffer (25 mM, pH 7.8), 0.5 mM oxidized GSH, 0.2 mM NADPH and the enzyme extract. The activity of GR was calculated by using extinction coefficient at 6.2 mM^{-1} cm^{-1}. One unit of enzyme was the amount necessary to decompose 1 μmol of NADPH min^{-1} at 25°C.

Protein content was determined following the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Determination of Content of Reduced Glutathione, Oxidized Glutathione, and Redox State

Glutathione was assayed by an enzymic recycling procedure of Anderson (1985). For specific assay of GSSG, the GSH was masked by derivatization with 2-vinylpyridine. Fresh leaves (500 mg) were homogenized in 2.0 mL of 5% sulphosalicylic acid under cold conditions. The homogenate was centrifuged at 10 000 × *g* for 10 min. A 300 μL aliquot of supernatant was taken and neutralized by addition of 18 μL 7.5 mol L^{-1} triethanolamine. One sample of 150 μL was then used to determine concentration of GSH plus GSSG. Another sample was pretreated with 3 μL 2-vinylpyridine for 60 min at 20°C to mask the GSH by derivatization, to allow the subsequent determination of GSSG alone. In each case, 50 μL aliquots of the two samples were mixed with 700 μL 0.3 mmol L^{-1} NADPH, 100 μL 5,5' -dithiobis-2-nitrobenzoic acid (DTNB) and 150 μL buffer containing 125 mmol L^{-1} sodium phosphate, 6.3 mmol L^{-1} EDTA (pH 6.5). A 10 μL aliquot of glutathione reductase (5 U mL^{-1}) was then added and the change in absorbance at 412 nm monitored at 30°C. Redox state was presented as the ratio of GSH to GSSG.

Assay of Leaf ATP-Sulfurylase and Sulfur Content

Activity of ATP-S (EC 2.7.7.4) was measured using method of Lappartient and Touraine (1996). Fresh leaf tissues (1.0 g) were ground at 4°C in a buffer consisting of 10 mM Na_2 EDTA, 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol (DTT), and 0.01 g mL^{-1} polyvinylpyrrolidone, using 1:4 (w/v) tissue to buffer ratio. The homogenate was centrifuged at 20,000 *g* for 10 min at 4°C. The supernatant was used for *in vitro* ATP-S assay. The reaction was initiated by adding 0.1 mL of extract to 0.5 mL of the reaction mixture, which contained 7 mM $MgCl_2$, 5 mM Na_2MoO_4, 2 mM Na_2ATP, and 0.032 units mL^{-1} of sulfate free inorganic pyrophosphate in 80 mM Tris-HCl buffer (pH 8.0). Another aliquot from the same extract was added to the same reaction mixture but without Na_2MoO_4. Incubations were carried out at 37°C for 15 min, after which phosphate was determined using UV-vis spectrophotometer.

Sulfur content was determined in leaf samples digested in a mixture of concentrated HNO_3 and 60% $HClO_4$ (85:1 v/v) using turbidimetric method of Chesnin and Yien (1950).

Determination of Leaf Nitrate Reductase Activity and Nitrogen Content

Activity of nitrate reductase (NR; EC 1.7.99.4) in leaf was measured by preparing enzyme extract using the method of Kuo et al. (1982). Leaves (1.0 g) were frozen in liquid N_2, ground to a powder with mortar and pestle and then stored at −80°C. The powder was thawed for 10 min at 4°C and homogenized in a blender with 250 mM Tris-HCl buffer (pH 8.5), containing 10 mM cysteine, 1 mM EDTA, 20 M FAD, 1 mM DTT, and 10% (v/v) glycerol. The homogenate was centrifuged at 10,000 *g* for 30 min at 4°C. Activity of NR was assayed spectrophotometrically as the rate of nitrite production at 28°C adopting the procedure of Nakagawa et al. (1984). The assay mixture contained KNO_3 (10 mM), HEPES (0.065 M; pH 7.0), NADH (0.5 mM) in phosphate buffer (0.04 mM; pH 7.2) and the enzyme in a final volume of 1.5 mL. The reaction was started by adding 0.5 mL NADH. After 15 min, the reaction was terminated by adding 1 mL of 1 N HCl solution containing 1% sulfanilamide followed by the addition of 1 mL of 0.02% aqueous *N*-1-napthylethylene-di-amine-dihydrochloride. The absorbance was read at 540 nm after 10 min.

Leaf N content was determined in acid-peroxide digested material using the method of Lindner (1944).

Determination of Photosynthetic-NUE and-SUE

Photosynthetic-NUE and -SUE were calculated by the ratio of net photosynthesis to N and S content per unit leaf area, respectively.

Measurement of PS II Activity

Fully expanded leaves were allowed to adapt under dark for 30 min before chlorophyll fluorescence measurements using Junior-PAM chlorophyll fluorometer (Heinz Walz, Germany) were made. Minimal fluorescence (Fo) and maximum fluorescence (Fm) were measured in dark-adapted leaves with a low measuring beam at a light intensity of 125 μmol m^{-2} s^{-1}, whereas under light-adapted condition, minimal fluorescence (Fo') and maximum fluorescence (Fm') were measured in the same leaves with a saturating light intensity (720 μmol m^{-2} s^{-1}) together with steady-state fluorescence (Fs). The variable fluorescence (F*v* and F*v*') was calculated using the values of F_m–F_o and Fm'–Fo', and actual PSII efficiency (Φ PS II) was determined as Fm'–Fs/Fm', maximal efficiency of PS II by using F*v*/Fm and intrinsic efficiency of PS II by using F*v*'/Fm'. Using fluorescence parameters determined in both the light- and dark-adapted states, the photochemical quenching (qP) and non-photochemical quenching (NPQ) parameters were calculated. Photochemical quenching was calculated as (Fm'–Fs)/F*v*' and NPQ as (Fm–Fm')/Fm' (Maxwell and Johnson, 2000). Electron

transport rate (ETR) was calculated by the following formula: Φ PSII × photosynthetic photon flux density × 0.5 × 0.84 as suggested by Krall and Edwards (1992).

Measurement of Leaf Gas Exchange Parameters

Gas exchange parameters (net photosynthesis, stomatal conductance, and intercellular CO_2 concentration) were measured in fully expanded uppermost leaves of plants using infrared gas analyzer (CID-340, Photosynthesis System, Bio-Science, USA). The measurements were done on a sunny day at light saturating intensity; PAR; 720 μmol m^{-2} s^{-1} and at 370 ± 5 μmol mol^{-1} atmospheric CO_2 concentrations.

Determination of Rubisco Activity and Chlorophyll Quantification

The activity of ribulose 1,5-bisphosphate carboxylase (Rubisco; EC 4.1.1.39) was determined adopting the method of Usuda (1985) by monitoring NADH oxidation at 30°C at 340 nm. For enzyme extraction, leaf tissue (1.0 g) was homogenized using a chilled mortar and pestle with ice-cold extraction buffer containing 0.25 M Tris-HCl (pH 7.8), 0.05 M $MgCl_2$, 0.0025 M EDTA, and 37.5mg DTT. This homogenate was centrifuged at 4°C at 10,000 *g* for 10 min. The resulting supernatant was used to assay the enzyme. The reaction mixture (3 mL) contained 100 mM Tris-HCl (pH 8.0), 40 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.2 mM NADH, 4 mM ATP, 5 mM DTT, 1 U of glyceraldehyde 3-phosphodehydrogenase, 1 U of 3-phosphoglycerate kinase and 0.2 mM ribulose 1,5-bisphosphate (RuBP).

Chlorophyll was extracted without maceration by cutting the leaves and incubating them in 30 mL of dimethyl sulfoxide (DMSO) and acetone mixture (1:1) in dark at 25°C for 30 min (Hiscox and Israelstam, 1979) and the content was quantified using the method of Arnon (1949).

Measurement of Growth Characteristics

Plants were uprooted, washed and dried on blotting paper, then were kept in an oven at 80°C till constant weight was obtained. The samples were weighed to obtain dry mass with the help of electronic balance. Leaf area was measured using a leaf area meter (LA211, Systronics, New Delhi, India).

Statistical Analysis

Data were analyzed statistically using analysis of variance (ANOVA) by SPSS 17.0 for Windows, and presented as treatment mean ± SE ($n = 4$). Least significant difference (LSD) was calculated for the significant data at $P < 0.05$. Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

RESULTS

Effect of Ni on Lipid Peroxidation, GSH Content, Net Photosynthesis, and Plant Dry Mass

The plants showed differential response to the applied Ni concentrations. The plants receiving lower Ni dose (50 mg Ni kg^{-1} soil) exhibited lower values of leaf TBARS content, whereas the higher Ni doses (100 and 200 mg Ni kg^{-1} soil) increased TBARS content in both the cultivars. The effect of 50 mg Ni kg^{-1} soil in reducing TBARS content was more pronounced in Varuna than RH30. The application of 50 mg Ni kg^{-1} soil reduced TBARS content by 14 and 10.8% in Varuna and RH30, respectively in comparison to control. In contrast, 100 and 200 mg Ni kg^{-1} soil showed more toxic effects; its application resulted in 61 and 147.6% increased TBARS content in Varuna, and 79.6 and 215% in RH30, respectively compared to control (**Table 1**). The increasing Ni concentrations increased GSH content in both the cultivars, but Varuna showed greater response than RH30 and exhibited highest GSH content with 200 mg Ni kg^{-1} soil compared to control. The application of 50, 100, and 200 mg Ni kg^{-1} soil resulted in increase of GSH content by 5.6, 9.5, and 19.4% in Varuna and 4.3, 8.6, and 16.7% in RH30, respectively compared to control (**Table 1**).

Application of 50 mg Ni kg^{-1} soil promoted net photosynthesis and plant dry mass whereas 100 and 200 mg Ni kg^{-1} soil significantly reduced in both the cultivars with greater reduction in RH30. Application of 50 mg Ni kg^{-1} soil increased net photosynthesis by 13.8% in Varuna and 18.8% in RH30 compared to control. However, the application of 100 and 200 mg Ni kg^{-1} soil reduced net photosynthesis by 15.8 and 8.2% in Varuna and 26.7 and 51.5% in RH30 compared to control (**Table 1**). Application of 50 mg Ni kg^{-1} soil increased

TABLE 1 | Thiobarbituric acid reactive substance (TBARS) content, GSH content, net photosynthesis, and plant dry mass of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS.

	Ni concentration (mg Ni kg^{-1} soil)							
	Control		50		100		200	
Parameters	Varuna	RH30	Varuna	RH30	Varuna	RH30	Varuna	RH30
TBARS content	21.4 ± 1.1^{g}	26 ± 1.0^{e}	18.4 ± 0.9^{h}	23.2 ± 1.18^{f}	34.5 ± 1.8^{d}	46.7 ± 2.4^{c}	53 ± 3.0^{b}	82 ± 4.0^{a}
GSH content	284 ± 9.7^{d}	210 ± 7^{h}	300 ± 11^{c}	219 ± 8.0^{g}	311 ± 13.8^{b}	228 ± 9^{f}	339 ± 15^{a}	245 ± 10^{e}
Net photosynthesis	15.2 ± 0.7^{b}	10.1 ± 0.6^{e}	17.3 ± 0.8^{a}	12 ± 0.6^{d}	12.8 ± 0.7^{c}	7.4 ± 0.4^{g}	9.4 ± 0.49^{f}	4.9 ± 0.3^{h}
Plant dry mass	5.1 ± 0.23^{b}	4.0 ± 0.2^{e}	5.8 ± 0.28^{a}	4.4 ± 0.21^{d}	4.7 ± 0.23^{c}	3.3 ± 0.16^{g}	3.6 ± 0.2^{f}	2.4 ± 0.17^{h}

Plants were basally treated with 0, 50, 100, and 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

plant dry mass by 13.7% in Varuna and 10% in RH30 compared to control. In contrast, application of 100 and 200 mg Ni kg^{-1} soil decreased plant dry mass by 7.8 and 29% in Varuna and 17.5 and 40% in RH30 compared to control (**Table 1**).

Effect of H_2O_2 on Lipid Peroxidation, GSH Content, Net Photosynthesis, and Plant Dry Mass

Application of 25 and 50 μM H_2O_2 reduced lipid peroxidation, while 100 μM H_2O_2 increased it. The treatment of 50 μM H_2O_2 was most effective in reducing lipid peroxidation in both the cultivars, but more prominently in Varuna. Lipid peroxidation was reduced by 42.9 and 28% in Varuna and RH30, respectively with 50 μM H_2O_2 in comparison to control. Application of 100 μM H_2O_2 proved toxic to both the cultivars and increased lipid peroxidation by 23.8% in Varuna and 28% in RH30 compared to control (**Table 2**). The increasing concentration of H_2O_2 increased GSH content, but the increase was more conspicuous in Varuna than RH30. Application of 25, 50, and 100 μM H_2O_2 increased GSH content by 19.6, 36, and 44.1% in Varuna and 9.1, 18.8, and 24.5% in RH30 compared to control (**Table 2**).

The varied effect of H_2O_2 concentration was noted on net photosynthesis and plant dry mass in both the cultivars. Application of 50 μM H_2O_2 improved net photosynthesis and plant dry mass more than 25 μM H_2O_2 in both the cultivars, with greater increase in Varuna than RH30. The application of 50 μM H_2O_2 increased net photosynthesis and plant dry mass by 29.2 and 21.2% in Varuna 17.3 and 20.6% in RH30 compared to control. In contrast, 100 μM H_2O_2 decreased net photosynthesis and plant dry mass in both the cultivars, with greater reduction in RH30 (**Table 2**).

Application of Hydrogen Peroxide Resisted Lipid Peroxidation and Electrolyte Leakage Induced by Ni Stress

Application of Ni increased lipid peroxidation (TBARS content) and electrolyte leakage in both the cultivars, but RH30 exhibited greater increase in TBARS content and electrolyte leakage. The increase in these parameters was 2.8- and 3.3-fold in Varuna, and 3.5- and 3.9-fold in RH30, respectively compared to control plants. Application of hydrogen peroxide reduced lipid peroxidation and electrolyte leakage in both the cultivars but to greater extent in Varuna than RH30 compared to control in no stress condition. Hydrogen peroxide-treated plants showed decrease in TBARS content and electrolyte leakage by 1.8- and 2.0-fold in Varuna, and by 1.3- and 1.6-fold in RH30 compared to control plants. Follow-up treatment of hydrogen peroxide to Ni grown plants resisted the peroxidation and electrolyte leakage resulting in lesser TBARS content and electrolyte leakage equally by 1.5-fold in Varuna and 1.1-fold in RH30 compared to Ni-treated plants (**Figure 1**).

Hydrogen Peroxide Accelerated Antioxidant System and Maintained Redox Status under Ni Stress

The effect of hydrogen peroxide was evaluated on enzymatic and non-enzymatic antioxidants system under Ni stress to assess the involvement of such defense mechanisms in Ni tolerance. The activity of antioxidant enzymes increased to a greater extent in Varuna than RH30. Application of hydrogen peroxide increased activity of APX and GR by 2.6- and 1.8-fold in Varuna and 2.0- and 1.3-fold in RH30 in plants grown without Ni, but by 3.5- and 1.3-fold in Varuna and 2.4- and 1.0-fold in RH30 in plants grown with Ni in comparison to control (**Figure 2**).

Plants grown with Ni showed increased GSH content more prominently in Varuna than RH30. Treatment with hydrogen peroxide in plants grown with Ni further increased the GSH content by 1.5-fold in Varuna and 1.1-fold in RH30 compared to control (**Figure 3**). However, plants grown under Ni stress showed higher GSSG (oxidized glutathione) content more prominently in Varuna than RH30. In contrast, H_2O_2-treated plants showed lesser value of GSSG compared to control plants. Redox state of Ni-treated plants reduced compared to control plants, but application of H_2O_2 to Ni-treated plants resulted in equal redox state to control plants (**Figure 3**).

Hydrogen Peroxide Increased N and S Assimilation and Photosynthetic-NUE and -SUE under Ni Stress

Under Ni stress, ATP-S activity increased while S content decreased in both the cultivars compared to control. In the

TABLE 2 | Thiobarbituric acid reactive substance content, GSH content, net photosynthesis, and plant dry mass of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS.

	H_2O_2 concentration (μM)							
	Control		25		50		100	
Parameters	Varuna	RH30	Varuna	RH30	Varuna	RH30	Varuna	RH30
TBARS content	21 ± 1.1^{c}	25 ± 1.0^{b}	17 ± 0.85^{d}	22 ± 1.1^{c}	12 ± 0.68^{e}	18 ± 1^{d}	26 ± 1.3^{b}	32 ± 1.6^{a}
GSH content	286 ± 11^{d}	208 ± 10^{h}	342 ± 16^{c}	227 ± 11^{g}	389 ± 17^{b}	247 ± 12^{f}	412 ± 19^{a}	259 ± 13^{e}
Net photosynthesis	15.4 ± 0.8^{c}	10.4 ± 0.7^{g}	17.3 ± 0.8^{b}	11.3 ± 0.6^{f}	19.9 ± 1.1^{a}	12.2 ± 0.5^{e}	13.1 ± 0.7^{d}	7.2 ± 0.4^{h}
Plant dry mass	5.2 ± 0.27^{c}	3.4 ± 0.21^{g}	5.7 ± 0.28^{b}	3.8 ± 0.21^{f}	6.3 ± 0.34^{a}	4.1 ± 0.23^{e}	4.5 ± 0.24^{d}	2.7 ± 0.16^{h}

Plants were basally treated with 0, 25, 50, and 100 μM H_2O_2 mg at 15 days after seed germination. Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

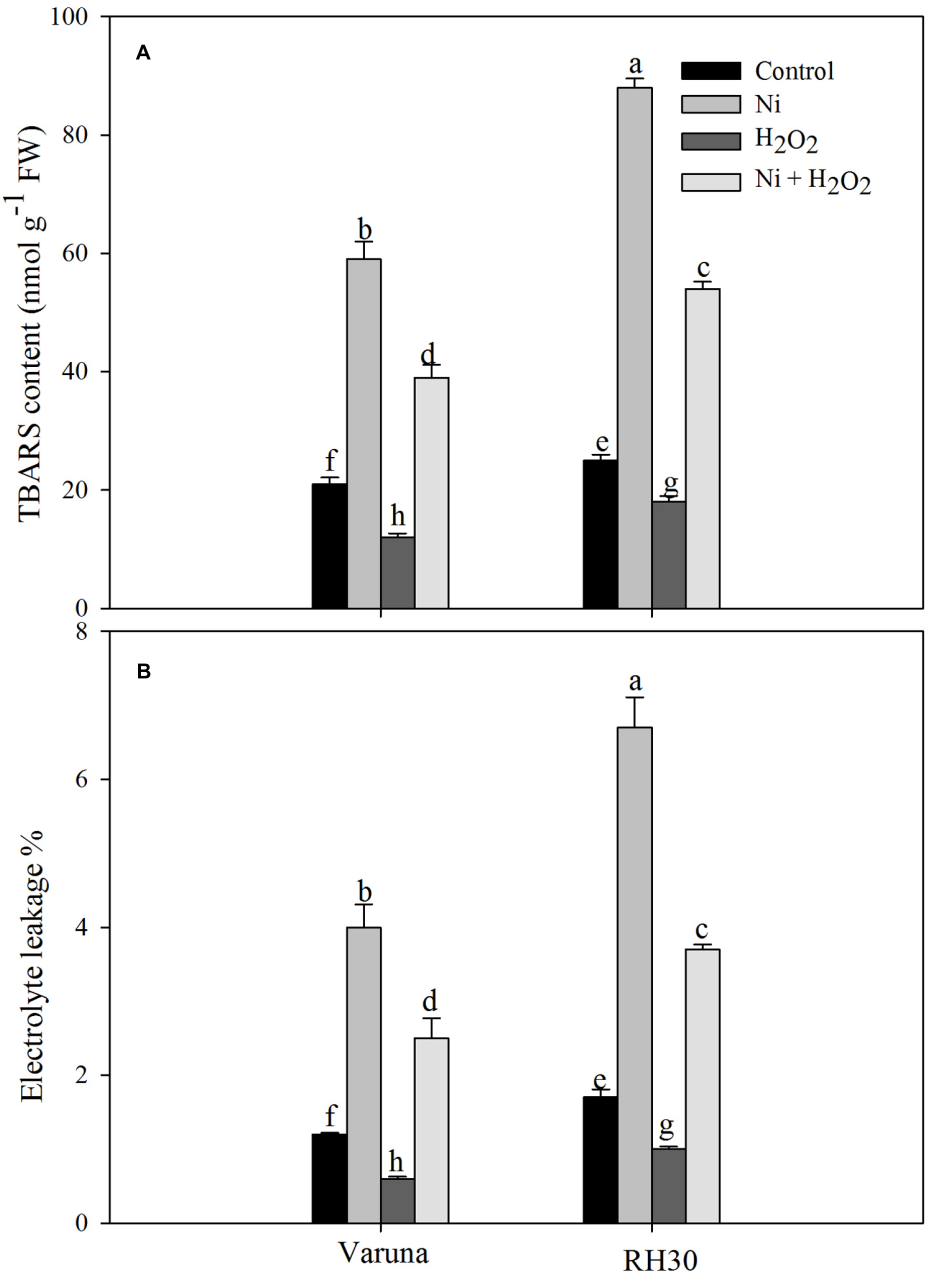

FIGURE 1 | Thiobarbituric acid reactive substance (TBARS) content (A) and electrolyte leakage (B) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

presence of Ni the increase in ATP-S activity was 23.5 and 15.3%, and decrease in S content was 31.1 and 21.9% in Varuna and RH30, respectively compared to control. Application of hydrogen peroxide increased ATP-S activity and S content by 68.8 and 21.3% in Varuna and 46.1 and 9.7% in RH30 compared to control in plants grown without Ni. In the presence of Ni, H_2O_2 application also resulted in increase in ATP-S activity and S content in both the cultivars (**Figure 4**).

Hydrogen peroxide application influenced N assimilation and S assimilation under no stress and Ni stress conditions. The application of hydrogen peroxide on plants grown with Ni proved beneficial in increasing the activity of NR and N content compared to control plants. Plants treated with Ni showed decrease in NR activity and N content by 23.6 and 33.5% in Varuna, and 33.4 and 41.2% in RH30, respectively compared to control plants. The decrease in NR activity and N content was

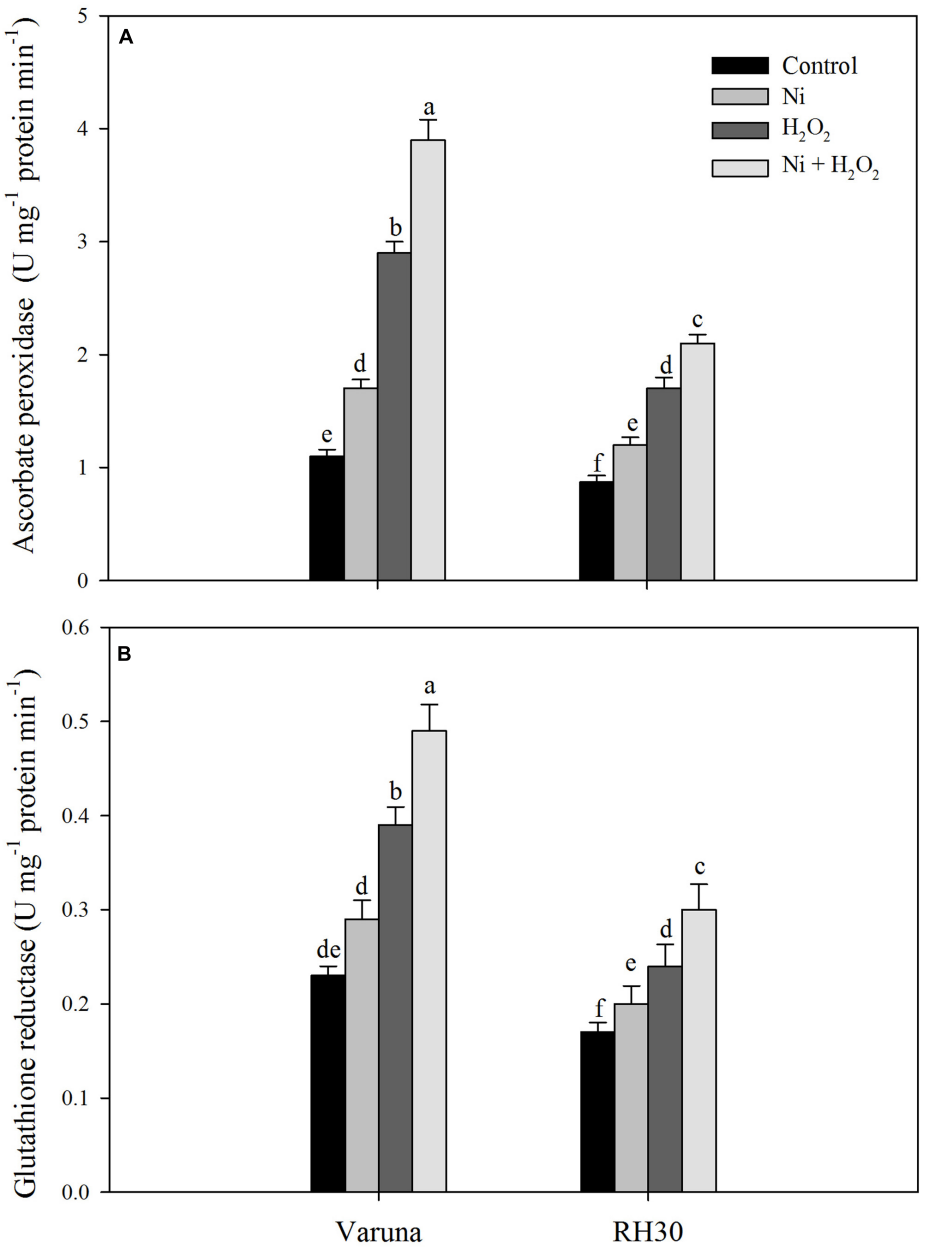

FIGURE 2 | Activity of ascorbate peroxidase (A) and glutathione reductase (B) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

reduced with hydrogen peroxide by 15.5 and 17.1% in Varuna and 23.1 and 24.6%, respectively in RH30 compared to control (**Figure 5**).

Photosynthetic-NUE and-SUE decreased more conspicuously in RH30 with Ni stress compared to control plants. The treatment of Ni decreased photosynthetic-NUE and -SUE by 35.7 and 32.2% in Varuna and 44.5 and 46.2% in RH30 compared to control plants. However, treatment with H_2O_2 reduced the toxic effect of Ni and increased photosynthetic-NUE and -SUE by 31.6 and 33.9% in Varuna and 19.0 and 13.9% in RH30, respectively compared to control (**Figure 6**).

Application of Hydrogen Peroxide Reversed Photosynthetic Inhibition by Ni

Gas exchange parameters were more severely affected in RH30 than Varuna plants under Ni stress. The reduction in net photosynthesis, stomatal conductance, and intercellular CO_2 concentration with Ni was 38.9, 27.9, and 27.6% in Varuna, while

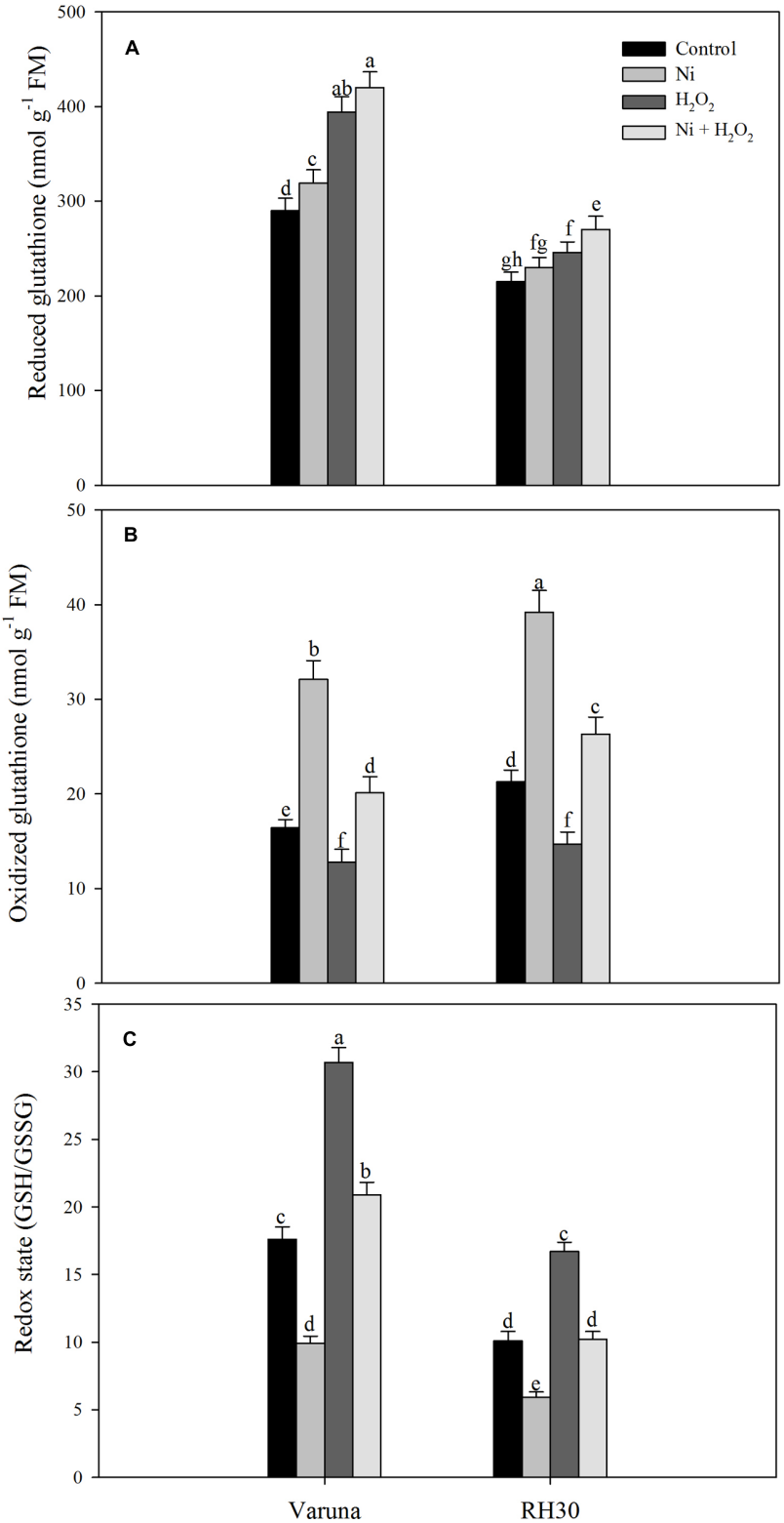

FIGURE 3 | Content of glutathione (GSH) reduced (A), GSH oxidized (GSSG; B) and redox state (GSH/GSSG; C) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

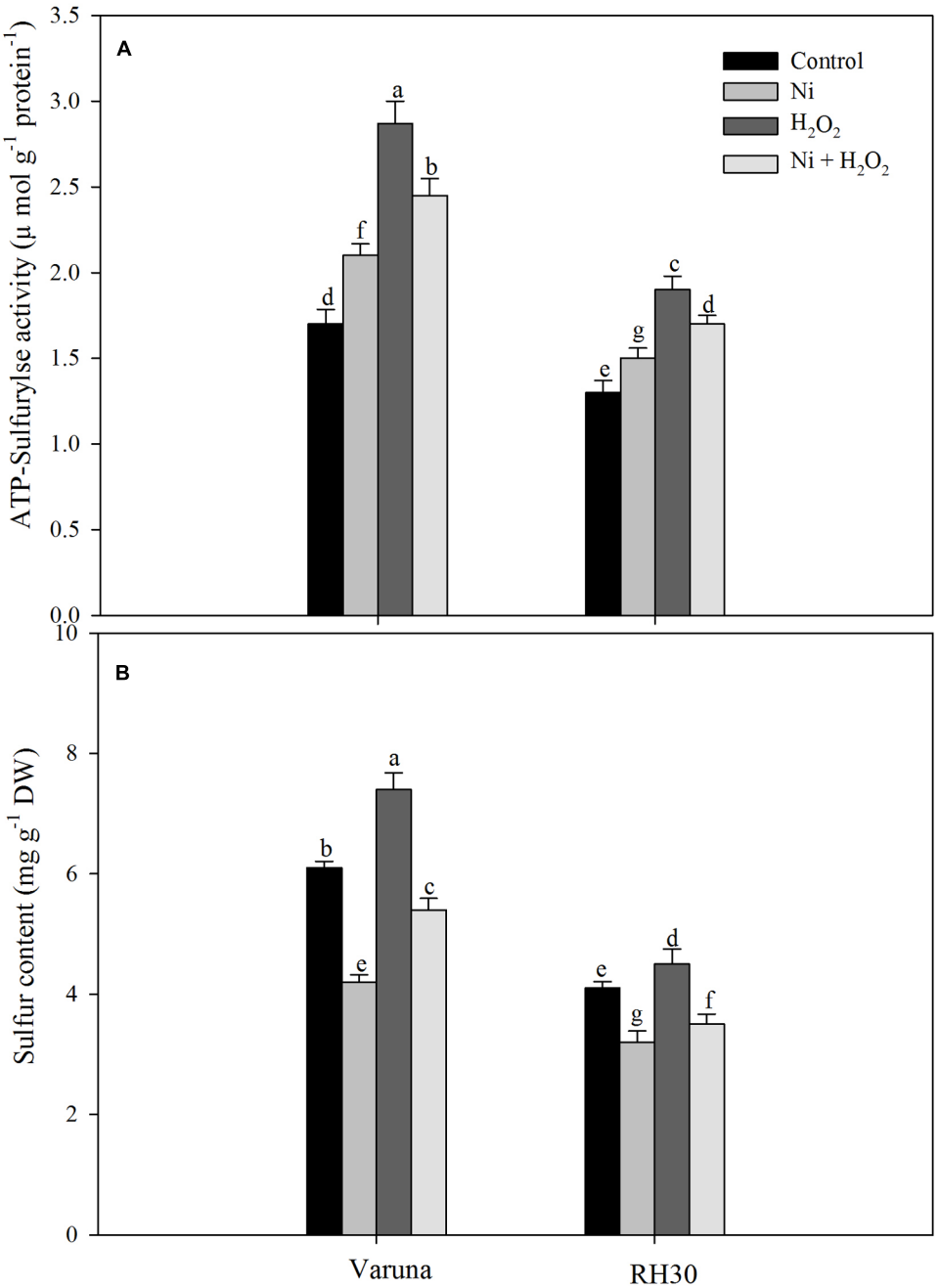

FIGURE 4 | Leaf ATP-sulfurylse activity (A) and sulfur content (B) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

these parameters decreased by 48.6, 39.7, and 41.6%, respectively in RH30 compared to control. Application of hydrogen peroxide increased net photosynthesis, stomatal conductance, and intercellular CO_2 concentration by 29.2, 18.2, and 25.0% in Varuna and by 14.0, 10.6, and 13.5%, respectively in RH30 in comparison to control. However, application of hydrogen peroxide to Ni-treated plants increased net photosynthesis, stomatal conductance, and intercellular CO_2 by 43.6, 28.7, and 30.3% in Varuna, and 30.9, 20, and 21.3% in RH30, respectively compared to Ni-treated plants (**Figure 7**).

Rubisco activity decreased more conspicuously in RH30 than Varuna compared to control under Ni treatment. However, hydrogen peroxide reduced the negative effects of Ni by increasing Rubisco activity in both the cultivars by increasing

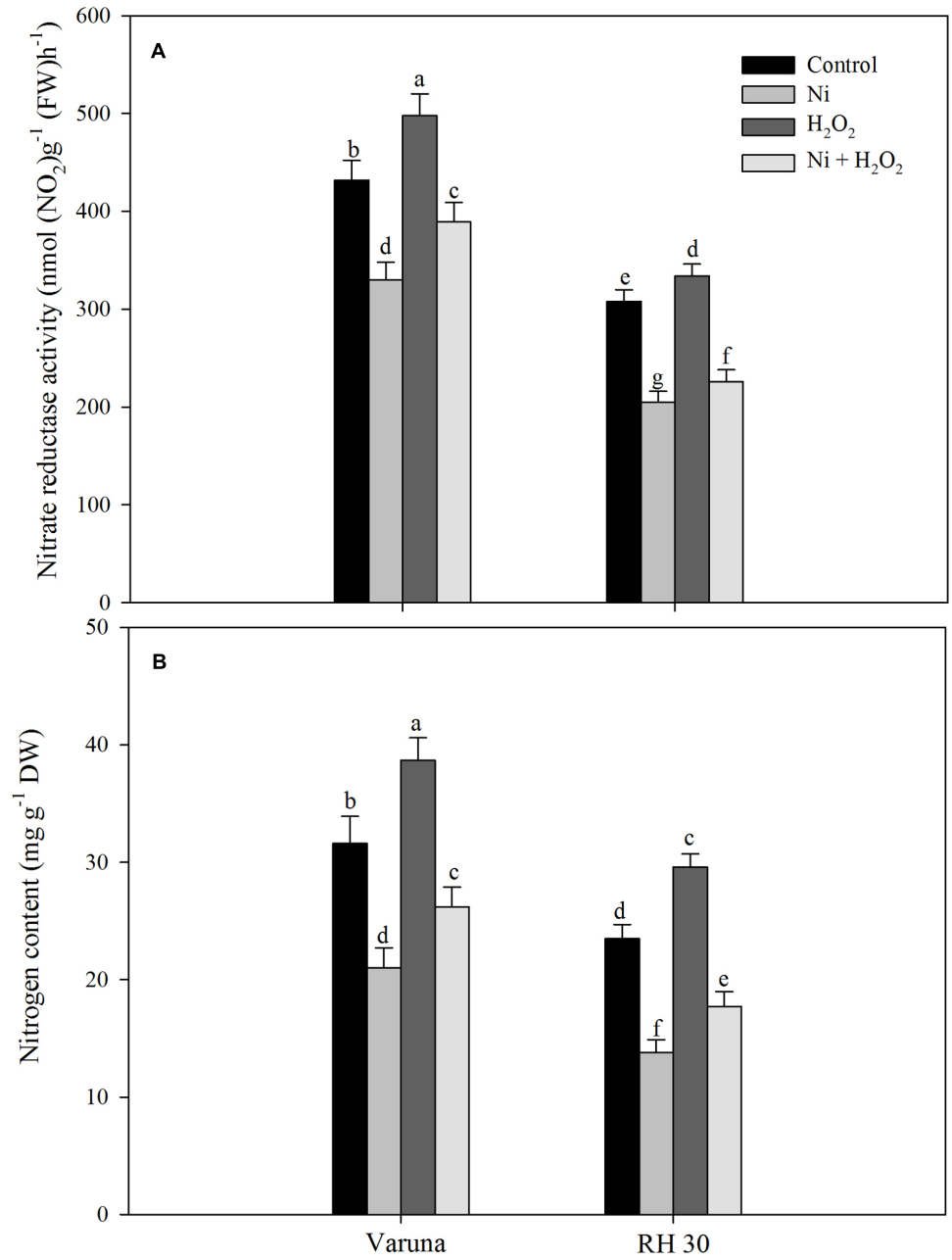

FIGURE 5 | Leaf nitrate reductase activity (A) and nitrogen content (B) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

the activity to 32.3% in Varuna and 22.7% in RH30 compared to control. Nickel toxicity also reduced chlorophyll content in both the cultivars more prominently in RH30 compared to control. The chlorophyll content was reduced by 23.4% in Varuna and 33.3% in RH30 with Ni compared to control. The content of chlorophyll increased by 28.3% in Varuna and 16.7% in RH30 in hydrogen peroxide-treated plant compared to control under no stress condition. However, in Ni-treated plants hydrogen peroxide treatment increased chlorophyll content by 23.4 and 16.3% in Varuna and RH30, respectively compared to Ni-treated plants (**Figure 8**).

Hydrogen Peroxide Modulated PS II Activity under Ni Stress

Chlorophyll fluorescence was measured in plants grown with Ni and treated with hydrogen peroxide. Plants grown with

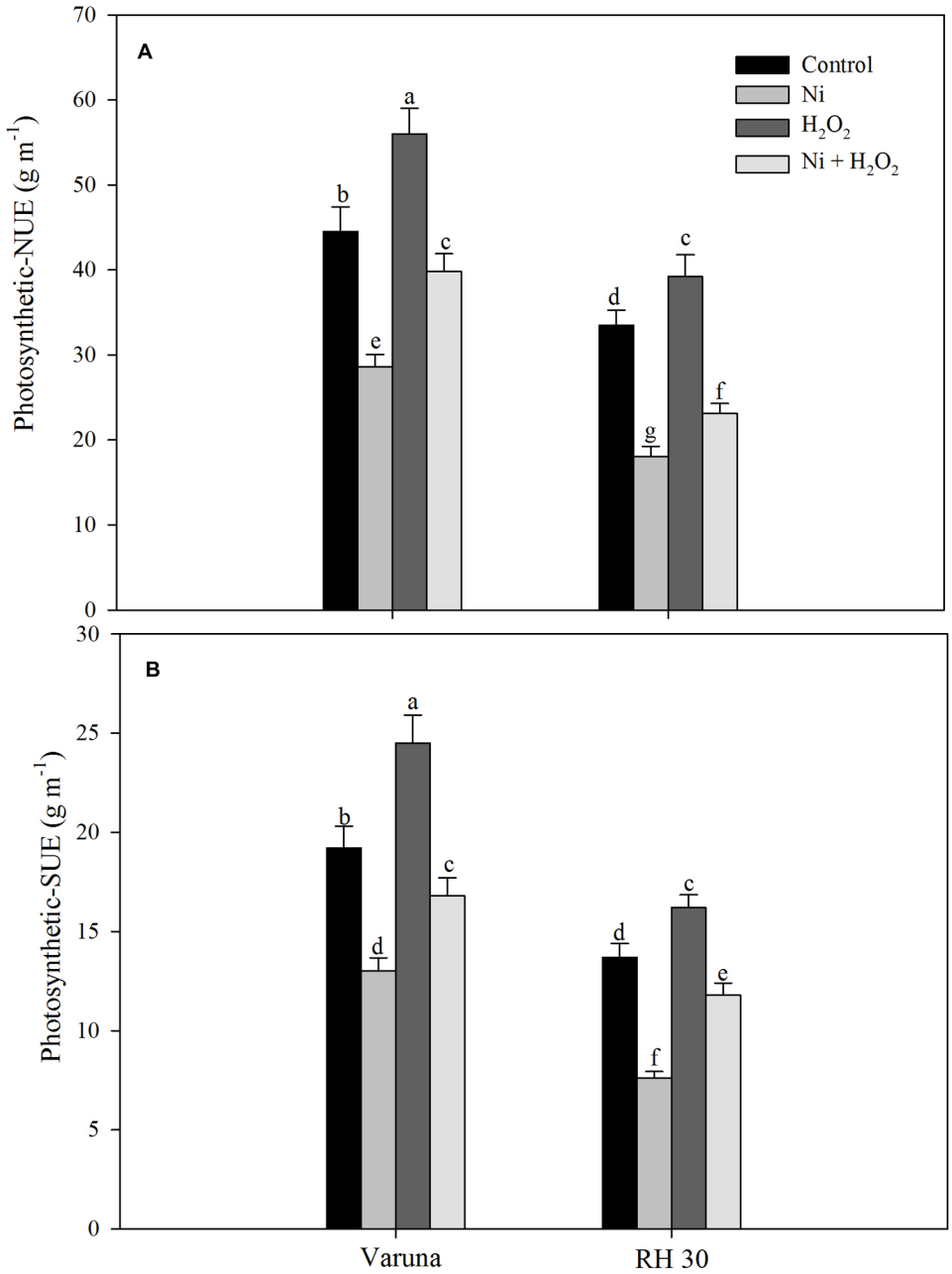

FIGURE 6 | Photosynthetic-nitrogen use efficiency (NUE; A) and photosynthetic-sulfur use efficiency (SUE; B) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

Ni exhibited reduced ΦPSII, Fv/Fm, intrinsic efficiency of PSII (Fv'/Fm'; **Figure 9**), ETR, and qP (**Figure 10**) compared to control. These attributes decreased by 19.7, 5.2, 7.4, 19.7, and 14.0% in Varuna and 18.5, 9.1, 10.6, 18.5, and 18.4%, respectively in RH30 in comparison to control plants. However, NPQ increased with Ni treatment by 84.6% in Varuna and by 95.7% in RH30 in comparison to control. Furthermore, NPQ significantly decreased with hydrogen peroxide application in Ni-treated plants in both the cultivars by 32.3 and 22.8%, respectively compared with Ni-treated plants. Follow-up treatment of H_2O_2 to Ni-treated plants proved effective in improving ΦPSII along with Fv/Fm, Fv'/Fm', ETR, and qP compared to Ni stress. Hydrogen peroxide application to plants with Ni stress inhibited the decrease and improved all the above characteristics by 22.4, 5.7, 8.3, 22.5, and 12.2% in Varuna and 16, 4.3, 6.7, 15.9, and 9.6% in

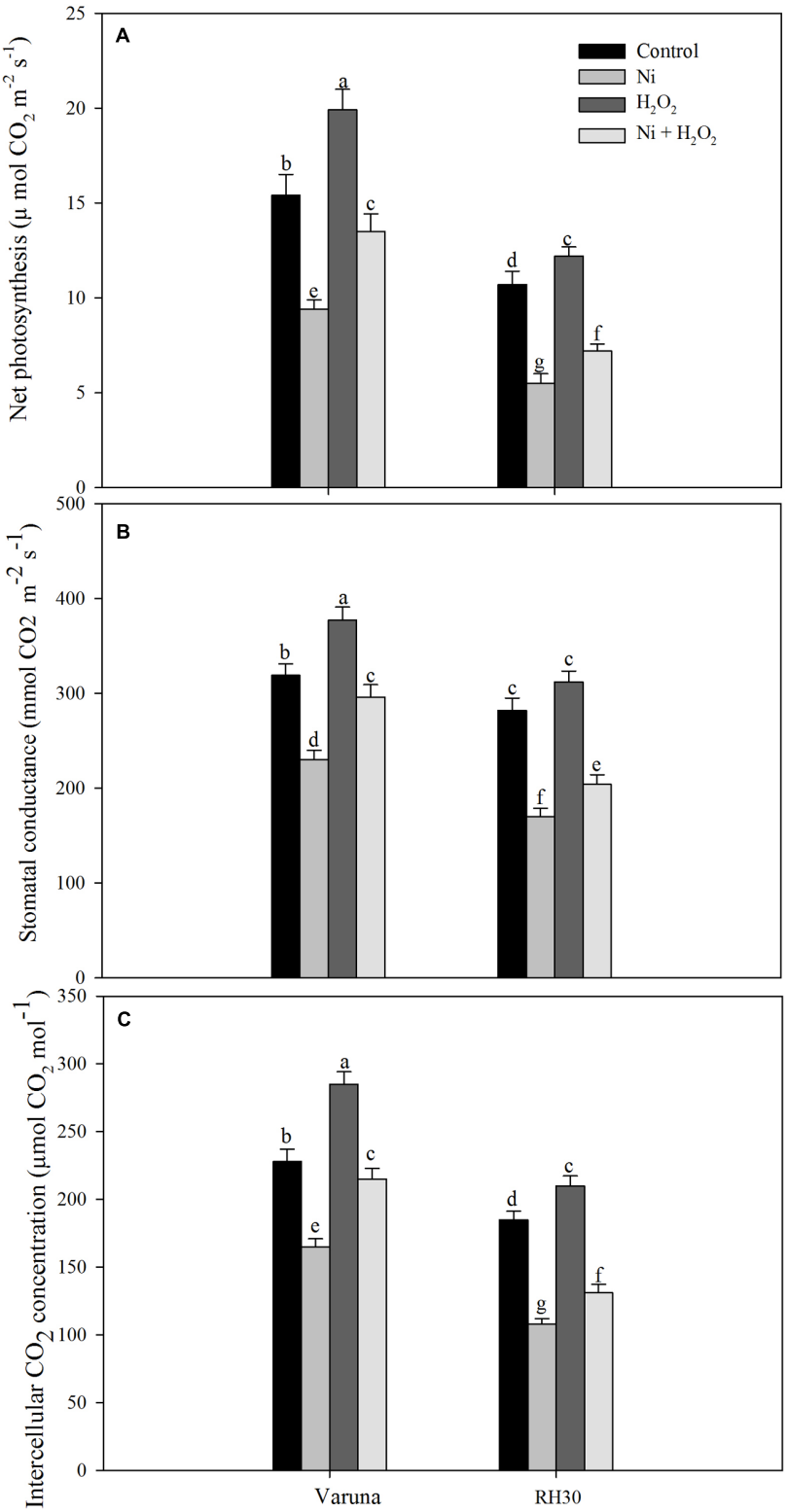

FIGURE 7 | Net photosynthesis (A) stomatal conductance (B) and intercellular CO_2 concentration (C) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

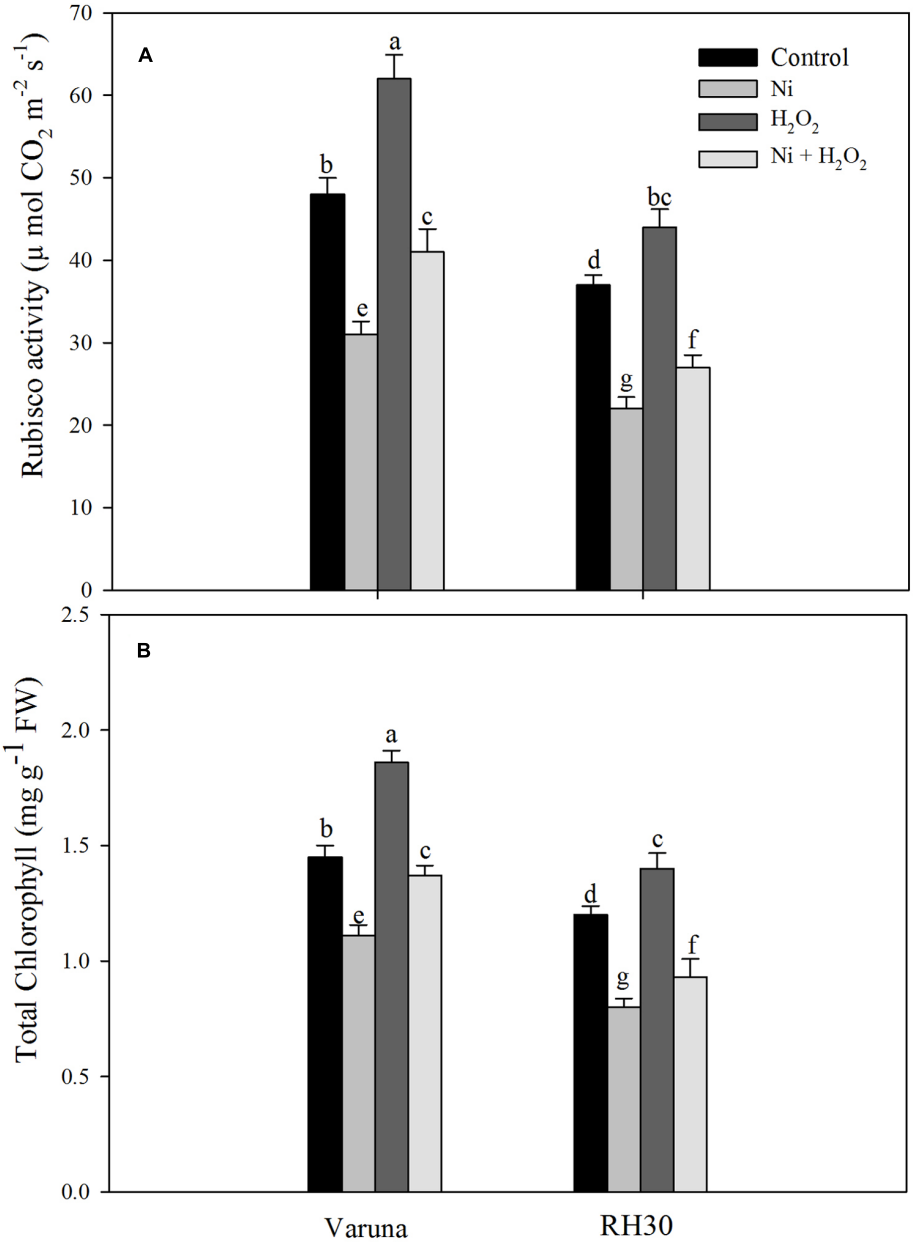

FIGURE 8 | Rubisco activity (A) and chlorophyll content (B) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

RH30, respectively compared with Ni-treated plants (**Figures 9** and **10**).

Hydrogen Peroxide Protected Growth Characteristics under Ni Stress

Leaf area and plant dry mass reduced with Ni treatment in both the cultivars, but more prominently in RH30 compared to control. Application of hydrogen peroxide resulted in reduction in adverse effects of Ni in both the cultivars and increased leaf area by 20.4% in Varuna and by 32.4% in RH30 and plant dry mass by 32.1% in Varuna and by 28.6% in RH30 as compared to Ni-treated plants. Under no stress, application of H_2O_2 increased leaf area and plant dry mass by 28.4 and 25.3% in Varuna and by 19.4 and 18.1% in RH30, respectively compared to the Ni treated plants (**Figure 11**).

A comparison of the percent changes in the observed parameters due to H_2O_2 in alleviation of Ni stress showed that the effect was more prominent in high photosynthetic cultivar Varuna than the low photosynthetic cultivar RH30 (**Table 3**).

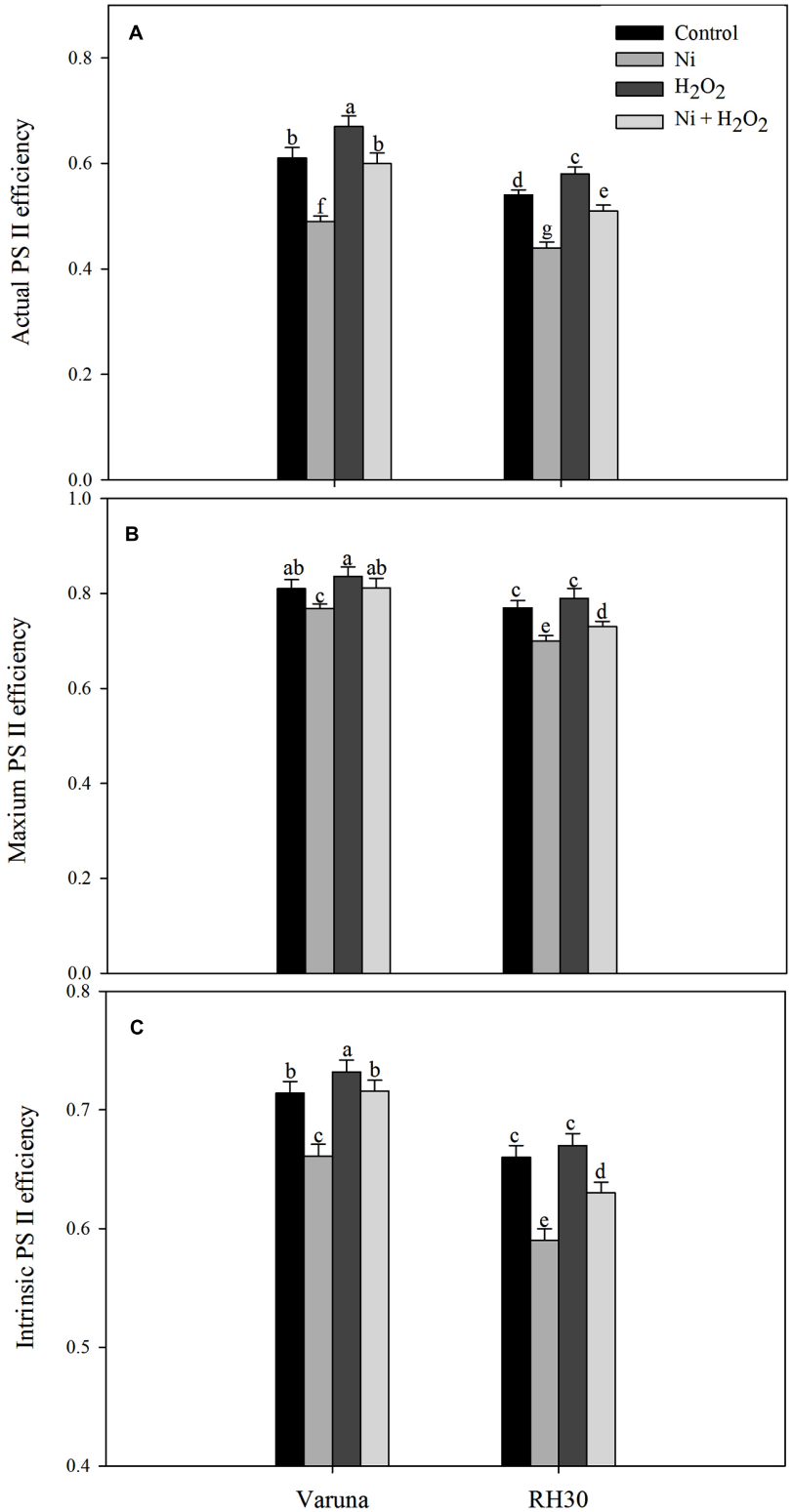

FIGURE 9 | Actual PS II efficiency (A) maximum PS II efficiency (B) and intrinsic PS II efficiency (C) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

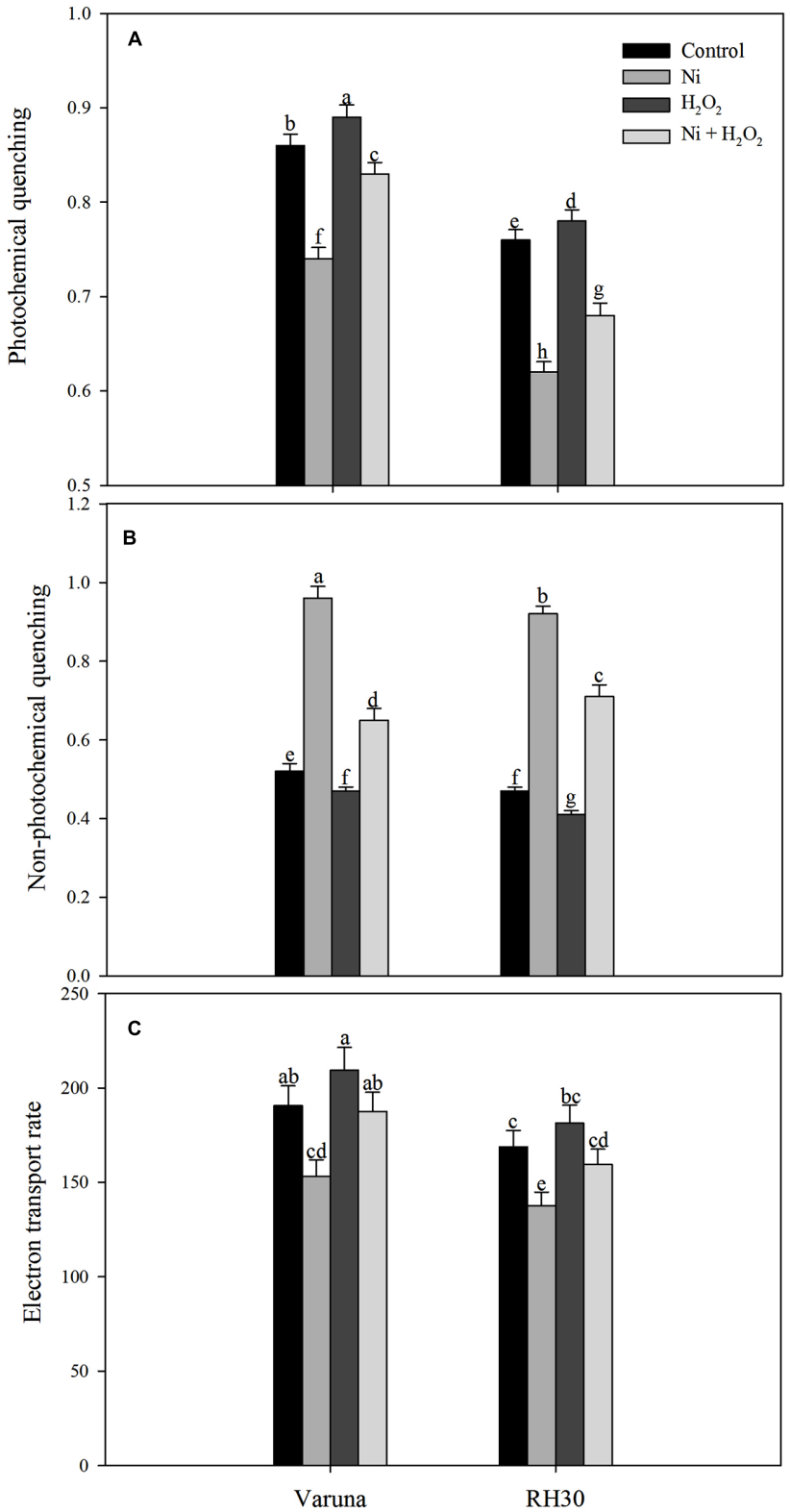

FIGURE 10 | Photochemical quenching (A) non-photochemical quenching (B) and electron transport rate (C) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

DISCUSSION

Hydrogen peroxide has been known as one of the factors responsible for generating oxidative stress in plant cells (Dat et al., 2000), but the recent studies have shown that it also acts as a signaling molecule participating in various response to abiotic stress tolerance (Gechev and Hille, 2005; Zhang et al., 2011; Zhou et al., 2012). It acts as a signaling molecule at lower concentrations, while provokes the onset of cell death at higher concentrations (Gechev and Hille, 2005). It regulates a number of physiological processes, such as acquisition of resistance, strengthening of cell wall, photosynthesis, and growth of plants (Cheeseman, 2007; Ślesak et al., 2007; Gao et al., 2010; Hossain et al., 2015). The requirement of H_2O_2 for better acclimation, improved survival and growth performances was shown for wheat (He et al., 2009) and rice (Hossain and Fujita, 2013) grown

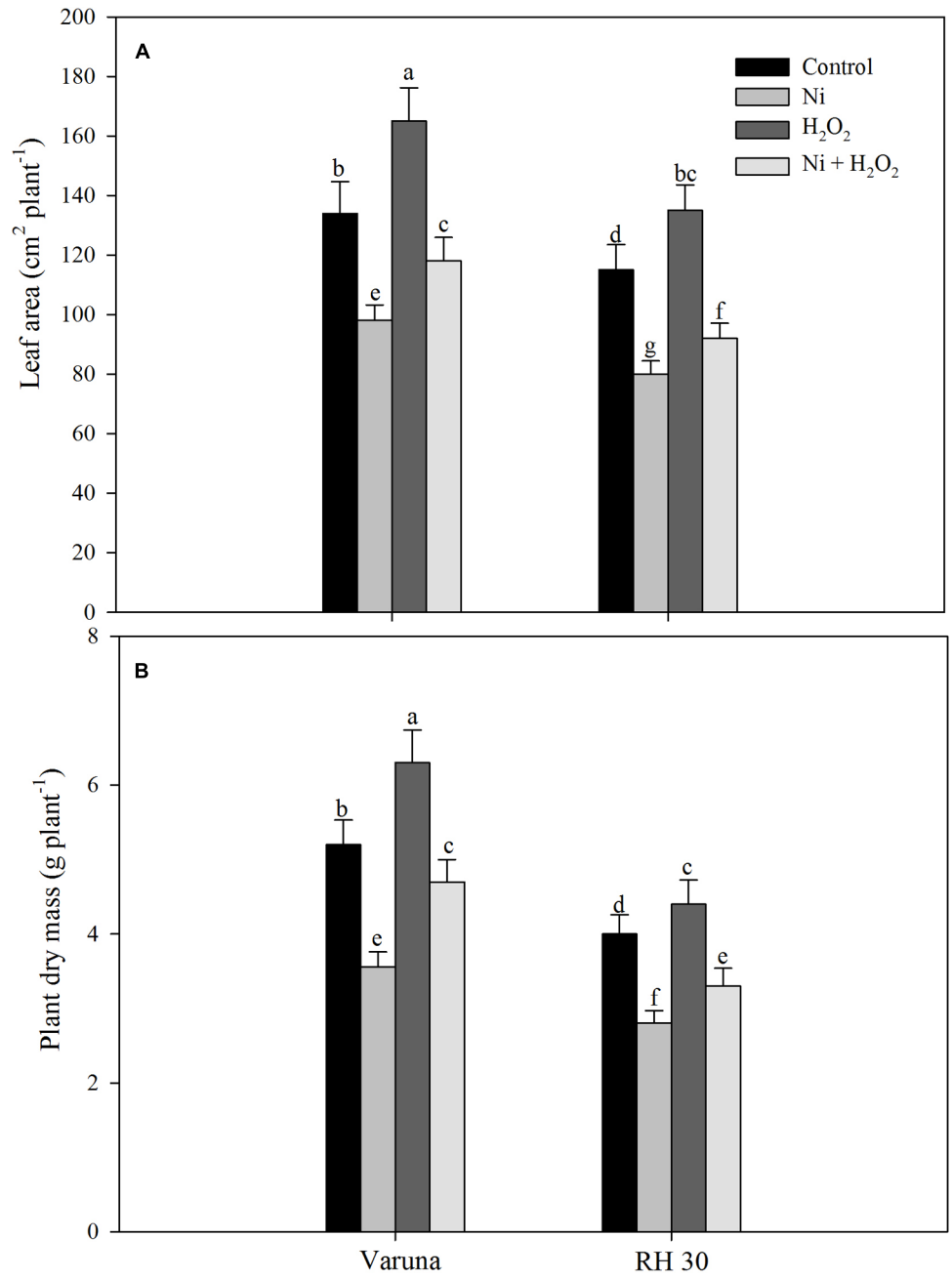

FIGURE 11 | Leaf area (A) and plant dry mass (B) of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS. Plants were basally treated with 0 or 50 μM H_2O_2 in presence or absence of 200 mg Ni kg^{-1} soil at 15 days after seed germination. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

under drought stress, for wheat (Wahid et al., 2007; Ashfaque et al., 2014) and rice (Uchida et al., 2002) grown under salt stress, for maize (Wahid et al., 2008) and rice (Uchida et al., 2002) grown under heat stress and mung bean plants under chilling stress (Yu et al., 2003).

Nickel is a micronutrient and plays important roles in plant metabolism. But, recent study of Khan and Khan (2014) have shown that high Ni concentration leads to excess production of ROS and reduction in photosynthesis and growth in mustard plants by reducing the activity of PS II and Rubisco. In the present study, it was found that Ni at 200 mg kg^{-1} soil proved toxic for photosynthesis and growth of both the mustard cultivars, apparently due to high oxidative stress as shown by TBARS content. It was also found that 50 μM H_2O_2 proved most effective in increasing photosynthesis and growth of mustard. Earlier reports also demonstrated that low H_2O_2 concentration improved salt and heat tolerance of rice plants by enhancing antioxidant system (Uchida et al., 2002) and salt tolerance in wheat by improved nutrient status (Wahid et al., 2007). Hossain and Fujita (2013) found that 50 μM H_2O_2 induced defense response in mustard seedlings by activation of methylglyoxal detoxification pathways under drought stress. Thus, our study is consistent with the previous studies showing detoxifying effects of H_2O_2 at low dose. Therefore, subsequent study was conducted by taking into account the involvement of high beneficial dose of H_2O_2 in modulation of photosynthetic responses in cultivars differing in photosynthetic capacity by monitoring its influence on oxidative stress, N and S-assimilation and photosynthetic-NUE and-SUE.

Nickel treatment increased TBARS content and electrolyte leakage in both the cultivars with greater values in RH30 than Varuna, but exogenously applied hydrogen peroxide lowered TBARS content and electrolyte leakage in both the cultivars with greater response in Varuna. In both the cultivars antioxidant defense system was induced upon exposure to Ni toxicity to avoid damage from generation of excess ROS. Application of H_2O_2 enhanced the activity of APX, GR and content of GSH more prominently in Varuna under Ni stress (**Table 3**) which helped in reducing TBARS content and electrolyte leakage. Moreover, more conspicuous increase in the activity of GR by H_2O_2 in Varuna

TABLE 3 | Percent changes in the parameters studied of Varuna (high photosynthetic capacity) and RH30 (low photosynthetic capacity) cultivars of mustard (*Brassica juncea* L.) at 30 DAS.

	1A		1B	
	H_2O_2 (compared to control)		Ni + H_2O_2 (compared to Ni)	
Parameters	**Varuna**	**RH30**	**Varuna**	**RH30**
TBARS content	75 ± 3.8* ↓	28 ± 1.6 ↓	33.9 ± 1.8* ↓	20.5 ± 1.2 ↓
Electrolyte leakage	50 ± 2.5* ↓	41 ± 2.2 ↓	37.5 ± 2.0* ↓	22.4 ± 1.3 ↓
Ascorbate peroxidase	163.6 ± 8.2* ↑	95.4 ± 4.9 ↑	129.4 ± 6.6* ↑	75 ± 3.9 ↑
Glutathione reductase	69.6 ± 3.5* ↑	41.2 ± 2.2 ↑	69 ± 3.6* ↑	50 ± 2.7 ↑
GSH content	35.9 ± 1.9* ↑	14.4 ± 0.9 ↑	31.7 ± 1.7* ↑	17.4 ± 0.9 ↑
GSSG content	22.0 ± 1.4* ↓	30.9 ± 1.7 ↓	37.4 ± 2.0* ↓	32.9 ± 1.8 ↓
Redox state	74.4 ± 3.8* ↑	65.3 ± 3.4 ↑	111.1 ± 5.8* ↑	72.9 ± 3.8 ↑
ATP-S activity	68.8 ± 3.5* ↑	46.2 ± 2.5 ↑	16.7 ± 0.9 ↑	13.3 ± 0.8 ↑
S content	21.3 ± 1.8* ↑	9.8 ± 0.6 ↑	28.6 ± 1.6* ↑	9.4 ± 0.6 ↑
NR activity	15.3 ± 0.9* ↑	8.4 ± 0.5 ↑	17.9 ± 0.9* ↑	10.2 ± 0.6 ↑
N content	22.5 ± 1.4 ↑	26 ± 1.5 ↑	24.8 ± 1.4 ↑	28.3 ± 1.5 ↑
Photosynthetic-NUE	25.8 ± 1.5* ↑	17.0 ± 1.1 ↑	39.2 ± 2.1 ↑	28.3 ± 2.2 ↑
Photosynthetic-SUE	27.6 ± 1.5* ↑	18.2 ± 1.0 ↑	29.2 ± 1.6* ↑	21 ± 1.1 ↑
Net photosynthesis	29.2 ± 1.7* ↑	14.1 ± 0.9 ↑	43.6 ± 2.3* ↑	30.9 ± 1.7 ↑
Stomatal conductance	18.2 ± 1.1* ↑	10.6 ± 0.6 ↑	28.7 ± 1.6* ↑	20 ± 1.2 ↑
Intercellular CO_2	25 ± 1.4* ↑	13.5 ± 0.8 ↑	30.3 ± 1.8* ↑	21.3 ± 1.4 ↑
Rubisco activity	29.2 ± 1.7* ↑	18.9 ± 1.3 ↑	32.3 ± 1.9* ↑	22.7 ± 1.5 ↑
Chlorophyll content	28.3 ± 1.7* ↑	16.7 ± 1.1 ↑	23.4 ± 1.3* ↑	16.3 ± 1.0 ↑
Actual PSII efficiency	9.8 ± 0.6 ↑	7 ± 0.4 ↑	22.4 ± 1.4 ↑	16 ± 1.0 ↑
Maximum PSII efficiency	3.2 ± 0.2 ↑	2.6 ± 0.3 ↑	5.7 ± 0.4 ↑	4.3 ± 0.4 ↑
Intrinsic PSII efficiency	2.5 ± 0.2 ↑	1.5 ± 0.2 ↑	8.3 ± 0.5 ↑	6.7 ± 0.4 ↑
Electron transport rate	9.9 ± 0.5 ↑	7.4 ± 0.4 ↑	22.5 ± 1.3 ↑	15.9 ± 1.0 ↑
Photochemical quenching	3.5 ± 0.2 ↑	2.6 ± 0.2 ↑	12.2 ± 0.8 ↑	9.6 ± 0.5 ↑
Non-photochemical quenching	9.6 ± 0.5 ↓	12.8 ± 0.8 ↓	32.3 ± 1.8 ↓	22.8 ± 1.9 ↓
Leaf area	23 ± 1.3* ↑	17.4 ± 1.1 ↑	20.4 ± 1.2* ↑	15 ± 0.9 ↑
Plant dry mass	21.2 ± 1.2* ↑	10 ± 0.6 ↑	32 ± 1.7* ↑	17.9 ± 1.1 ↑

*Data are presented as treatments mean ± SE (n = 4). Data followed by * shows significant difference between the two cultivars by LSD test at P < 0.05. Symbols ↑ and ↓ represent increase and decrease, respectively.*

indicated higher amount of GSH production in this cultivar to eliminate the products of lipid peroxidation. The activity of GR has been reported to be associated with alteration of the cellular redox status and decisive in determining plant resistance to Ni stress (Khan and Khan, 2014). Also, GR maintains homeostasis of GSH and GSSG crucial for signaling stress response and regulating oxidative stress. Exogenous application of H_2O_2 has been shown to promote the expression of stress-response genes and increase Ni stress tolerance. Gao et al. (2010) found that exogenously sourced H_2O_2 induced antioxidative enzymes, GR, DHAR, and MDHAR under heat stress condition. The increase in tolerance to abiotic stresses such as heat, chilling, and salts by exogenous application of H_2O_2 has been reported in different plants through increase in the activity of antioxidants and reducing peroxidation of membrane lipids (Uchida et al., 2002; de Azevedo Neto et al., 2005; Wahid et al., 2007). Up-regulation of antioxidative defense system by passive absorption of H_2O_2 in germinating seeds has been shown to offset oxidative damage leading to improved physiological attributes (Wahid et al., 2007, 2008). The other aspect of increase in GSH content by H_2O_2 in both the cultivars under Ni stress was the increase in ATP-S activity. It has been reported that increased GSH content counteracted the Cd-induced oxidative stress with lesser damages to photosynthesis in mustard (Masood et al., 2012; Asgher et al., 2014).

In the present study, application of H_2O_2 increased photosynthesis of plants treated with Ni largely because of increase in the activity of Rubisco and PS II. However, the smaller increase in stomatal conductance and intercellular CO_2 concentration in these plants may not wholly account for the larger increase in photosynthesis, suggesting the involvement of non-stomatal limitations as well for the increase in net photosynthesis. These effects were more pronounced in high photosynthetic capacity cultivar Varuna than the low photosynthetic capacity cultivar RH30. The demand for N and S increased under Ni stress and increased the utilization of N and S more effectively in Varuna than RH30 and proved more beneficial in the alleviation of Ni-induced oxidative stress and protection of photosynthesis under Ni stress condition. The increased N and S assimilation resulting from H_2O_2 application accounted for higher NUE and SUE that caused greater photosynthesis through its incorporation into Rubisco more conspicuously in Varuna. The increase in N and S assimilation contributes to chlorophyll biosynthesis, Rubisco activity and in the regulation of photosynthesis (Marschner, 1995). Deprivation of either N or S may cause a significant reduction in the photosynthetic efficiency of plants (Resurreccion et al., 2001; Lunde et al., 2008). The relationship between N content and photosynthesis has been observed in mustard plants (Iqbal et al., 2011), which is also correlated with Rubisco content and S assimilation (Sexton et al., 1998; Iqbal et al., 2012). Higher allocation of N to leaf through the increase in the activity of NR with application of H_2O_2 under Ni stress in Varuna increased photosynthesis. It has been suggested that plants with lower photosynthetic-NUE have a lower ability to allocate N to the photosynthetic machinery (Takashima et al., 2004). However, reports on the influence of H_2O_2 on photosynthetic-NUE and -SUE in cultivars differing in photosynthetic capacity and grown with Ni in the two cultivars are not known. In the present study, H_2O_2 treatment increased net photosynthesis in Varuna greater than RH30 through the greater increase in N and S assimilation and photosynthetic-NUE and -SUE under Ni stress (**Table 3**). Li et al. (2011) observed that exogenous H_2O_2 treatment decreased the deleterious effect of salt stress on growth of wheat.

The decrease in chlorophyll content with Ni treatment possibly decreased the absorption of light by the chloroplast and thus indirectly impaired photosynthesis. Photosystem II (PS II) is uniquely vulnerable to the damage by metal stress and chlorophyll fluorescence parameters are a reliable indicator of the intensity of abiotic stress. Chlorophyll fluorescence parameters such as actual efficiency of PS II (ΦPSII), maximum efficiency of PS II (Fv/Fm), and intrinsic efficiency of PS II (Fv'/Fm') are related to photosynthetic efficiency (Maxwell and Johnson, 2000). Here we found that RH30 was more prone to Ni stress than Varuna as shown by greater decrease in chlorophyll fluorescence parameters (ΦPSII, Fv/Fm, Fv'/Fm', ETR, and qP) in RH30, and the observed reduction in photosynthesis was mainly attributable to photo-inhibition during the Ni stress. In contrast, NPQ increased under Ni stress with prominent increase in RH30 than Varuna. Ni-induced photo-inhibition and reduced photosynthetic efficiency was reversed by the exogenously sourced H_2O_2. The present work demonstrated that application of H_2O_2 caused up-regulation of antioxidant system (APX and GR activity and GSH content) and mitigated oxidative damage caused by Ni. The alleviation of oxidative stress by H_2O_2 is reflected by significant improvement in early growth characteristics of mustard cultivars most pronounced in high photosynthetic potential cultivar (Varuna) than low photosynthetic potential cultivar (RH30; **Table 3**). The outcome of the study as considering H_2O_2 as signaling molecule will help in developing an agricultural friendly technique to overcome the effects of Ni stress on photosynthesis and plant dry mass.

CONCLUSION

Conclusively, it may be said that low concentration of H_2O_2 induces Ni tolerance by avoiding oxidative stress through increased capacity for N and S assimilation with greater photosynthetic-NUE and -SUE more prominently in Varuna than RH30. The induced activity of ATP-S and antioxidant enzymes (APX and GR) by H_2O_2 helped in higher GSH production in Varuna than RH30 and reversed the Ni stress more efficiently. These characteristics of Varuna helped in protecting photosynthesis and maintaining high plant dry mass than RH30. The lower induction of photosynthesis by H_2O_2 in RH30 was related to a lesser N and S assimilation and lesser GSH production. The information on the physiological response of mustard in relation to N and S assimilation differing in photosynthetic potential cultivars could be used in understanding the key role of H_2O_2 in increasing photosynthesis and growth under Ni stress. Moreover, studies should be focussed to unravel the

positive role of H_2O_2 in increasing photosynthesis utilizing molecular tools.

AUTHOR CONTRIBUTIONS

MK designed the experiment, carried out the analyses and prepared the manuscript. NK supervised the work and was involved in the design of the experiment, preparation, and presentation of the manuscript. AM, TP, and MA carried out the experimental work and searched literature for the work on which this article is based.

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REVIEW
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Different Modes of Hydrogen Peroxide Action During Seed Germination

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Hydrogen peroxide was initially recognized as a toxic molecule that causes damage at different levels of cell organization and thus losses in cell viability. From the 1990s, the role of hydrogen peroxide as a signaling molecule in plants has also been discussed. The beneficial role of H_2O_2 as a central hub integrating signaling network in response to biotic and abiotic stress and during developmental processes is now well established. Seed germination is the most pivotal phase of the plant life cycle, affecting plant growth and productivity. The function of hydrogen peroxide in seed germination and seed aging has been illustrated in numerous studies; however, the exact role of this molecule remains unknown. This review evaluates evidence that shows that H_2O_2 functions as a signaling molecule in seed physiology in accordance with the known biology and biochemistry of H_2O_2. The importance of crosstalk between hydrogen peroxide and a number of signaling molecules, including plant phytohormones such as abscisic acid, gibberellins, and ethylene, and reactive molecules such as nitric oxide and hydrogen sulfide acting on cell communication and signaling during seed germination, is highlighted. The current study also focuses on the detrimental effects of H_2O_2 on seed biology, i.e., seed aging that leads to a loss of germination efficiency. The dual nature of hydrogen peroxide as a toxic molecule on one hand and as a signal molecule on the other is made possible through the precise spatial and temporal control of its production and degradation. Levels of hydrogen peroxide in germinating seeds and young seedlings can be modulated via pre-sowing seed priming/conditioning. This rather simple method is shown to be a valuable tool for improving seed quality and for enhancing seed stress tolerance during post-priming germination. In this review, we outline how seed priming/conditioning affects the integrative role of hydrogen peroxide in seed germination and aging.

Keywords: dormancy, germination, hydrogen peroxide, phytohormone, priming, reactive oxygen species, seed, signaling molecule

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INTRODUCTION

Hydrogen peroxide (H_2O_2) is a reactive molecule that plays a dual role in plant physiological and developmental processes and in resisting stress. The mutual relationship between positive and negative functions performed by H_2O_2 in biological systems depends on the H_2O_2 concentration, on physiological conditions, and on the specificities of processes affected by H_2O_2. Thus, it is challenging to clearly distinguish between beneficial (signaling) and deleterious (causing damage)

roles played by H_2O_2. It is also a considerable challenge to separate the roles of H_2O_2 from those of other reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($^{\bullet}OH$), which may coexist and be converted into one another through spontaneous and catalyzed reactions. In this review, we focus on functions performed by H_2O_2 during seed germination and their modulation as a result of pre-sowing seed priming.

Seed germination is one of the most important stages of the plant life cycle. The efficient progression of germination determines the nature of seedling establishment and the proper development of mature plants. Germination is a very complex process that begins with water uptake and involves events associated with the transition of a quiescent dry seed to a metabolically active state. The emergence of the embryonic axis through structures surrounding the embryo is considered to be a final stage of germination (Weitbrecht et al., 2011; Bewley et al., 2013). Key processes associated with germination involve the reactivation of metabolism, the resumption of cellular respiration, the biogenesis of mitochondria, DNA repair, the translation and/or degradation of stored mRNAs, the transcription and translation of new mRNAs, and the onset of reserve mobilization (Bentsik and Koornneef, 2008; Nonogaki et al., 2010; Bewley et al., 2013).

These biochemical and cellular events triggered by water uptake are accompanied by the generation of ROS (especially H_2O_2) as shown in **Figure 1** (El-Maarouf-Bouteau and Bailly, 2008). The accumulation of H_2O_2 and of other ROS has been identified in seed physiology during imbibition and during early stages of germination, mainly as a result of a pronounced increase in their intracellular and extracellular production (Schopfer et al., 2001; Kranner et al., 2010; Zhang et al., 2014b; Kubala et al., 2015b). While ROS are also produced in dry seeds, they (or at least H_2O_2) fulfill their functions as cellular messengers or toxic molecules, mainly when seeds become hydrated, i.e., during imbibition and germination (Bailly et al., 2008). A comparative study on water uptake, on its distribution and on associated free radical and H_2O_2 production was conducted in reference to pea imbibition and germination (*Pisum sativum*) seeds (Wojtyla et al., 2006). ROS are often recognized as a main source of seed deterioration associated with a loss of seed vigor and as a repercussion of aging (Kumar et al., 2015). At a hydrated state, an intense increase in respiratory activity spurs superoxide anion production during electron leakage from the mitochondrial electron transport chain followed by dismutation to H_2O_2.

Other sources of ROS are NADPH oxidases of the plasma membrane, also known as respiratory burst oxidase homologs (Rboh), and extracellular peroxidases, which can produce superoxide radicals that are subsequently converted to H_2O_2. Glyoxysomes also participate in the intense production of H_2O_2 via the β-oxidation pathway during oil reserve mobilization (Bailly, 2004; El-Maarouf-Bouteau and Bailly, 2008). Among ROS, H_2O_2, a long-lived ROS that can diffuse easily through membranes and that can reach targets far from production sites, is recognized as an important signaling molecule (Møller et al., 2007). However, H_2O_2 has strong oxidizing capacities that render it capable of interacting with most biomolecules (including nucleic acids, proteins, and lipids), thus resulting in oxidative stress that causes cellular damage. Lipid peroxidation is one of the most widely documented toxic effects of H_2O_2 on cellular components and biological molecules. Lipid peroxidation affects polyunsaturated fatty acids (PUFAs) found in cell membranes or reserve lipids. Nucleic acids (DNA, RNA) and proteins are also potential targets of oxidation by H_2O_2 (El-Maarouf-Bouteau and Bailly, 2008). Oxidative DNA damage induced by H_2O_2 leads to the accumulation of 7,8-dihydro-8-oxoguanine (8-oxo-dG), which has been shown to cause the accumulation of double-strand breaks in genome and deleterious effects on cell viability (Pommier et al., 2003).

DNA oxidation by ROS is considered a main source of DNA damage during seed storage and germination. Recently published data have shown that mRNA is much more sensitive to oxidative damage than DNA, mainly due to its cellular localization, single stranded structure and lack of repair mechanisms (Kong and Lin, 2010). As in DNA, the most frequently oxidized base in RNA is guanine, from which oxidation leads to the accumulation of 8-hydroxyguanosine (8-OHG). Oxidative damage to mRNA results in the inhibition of protein synthesis and in protein degradation (El-Maarouf-Bouteau et al., 2013; Chmielowska-Bąk et al., 2015). Protein oxidation can alter protein functions as a result of modifications made to their enzymatic and binding properties (Davies, 2005). Indeed, H_2O_2 accumulation and associated oxidative damages together with a decline in antioxidant mechanisms can be regarded as a source of stress that may affect the successful completion of germination. However, H_2O_2 is also regarded as a signaling hub for the regulation of seed dormancy and germination, and the precise regulation of H_2O_2 accumulation by cell antioxidant machinery is crucial to achieve a balance between oxidative signaling that promotes germination and oxidative damage that prevents or delays germination. These findings were clearly summarized and presented as the principle of the "oxidative window" for germination by Bailly et al. (2008). According to this hypothesis, both lower and higher levels of ROS impair seed germination, and this is only possible within a critical range of concentrations.

Recent evidence shows that the selective oxidation of proteins and mRNAs can act as a positive regulator of seed germination (Job et al., 2005; Oracz et al., 2007; Barba-Espín et al., 2011; Bazin et al., 2011). Bazin et al. (2011) showed that approximately 24 stored mRNAs undergo oxidation during sunflower (*Helianthus annuus*) after ripening. Most of these transcripts correspond to proteins involved in cellular signaling. Moreover, the same authors showed that 8-OHG levels increase in mRNA by 50% during dormancy alleviation. Job et al. (2005) observed massive protein oxidation processes during *Arabidopsis thaliana* seed germination. These authors found that mainly reserve proteins (12S subunits of cruciferin) are oxidized during seed maturation and that the same proteins gradually degrade during imbibition. Similar observations were made by Barba-Espín et al. (2011) through their research on pea seed germination. These authors also reported reserve protein carbonylation processes, i.e., vicilins and albumin 2. The oxidation of seed storage proteins during seed

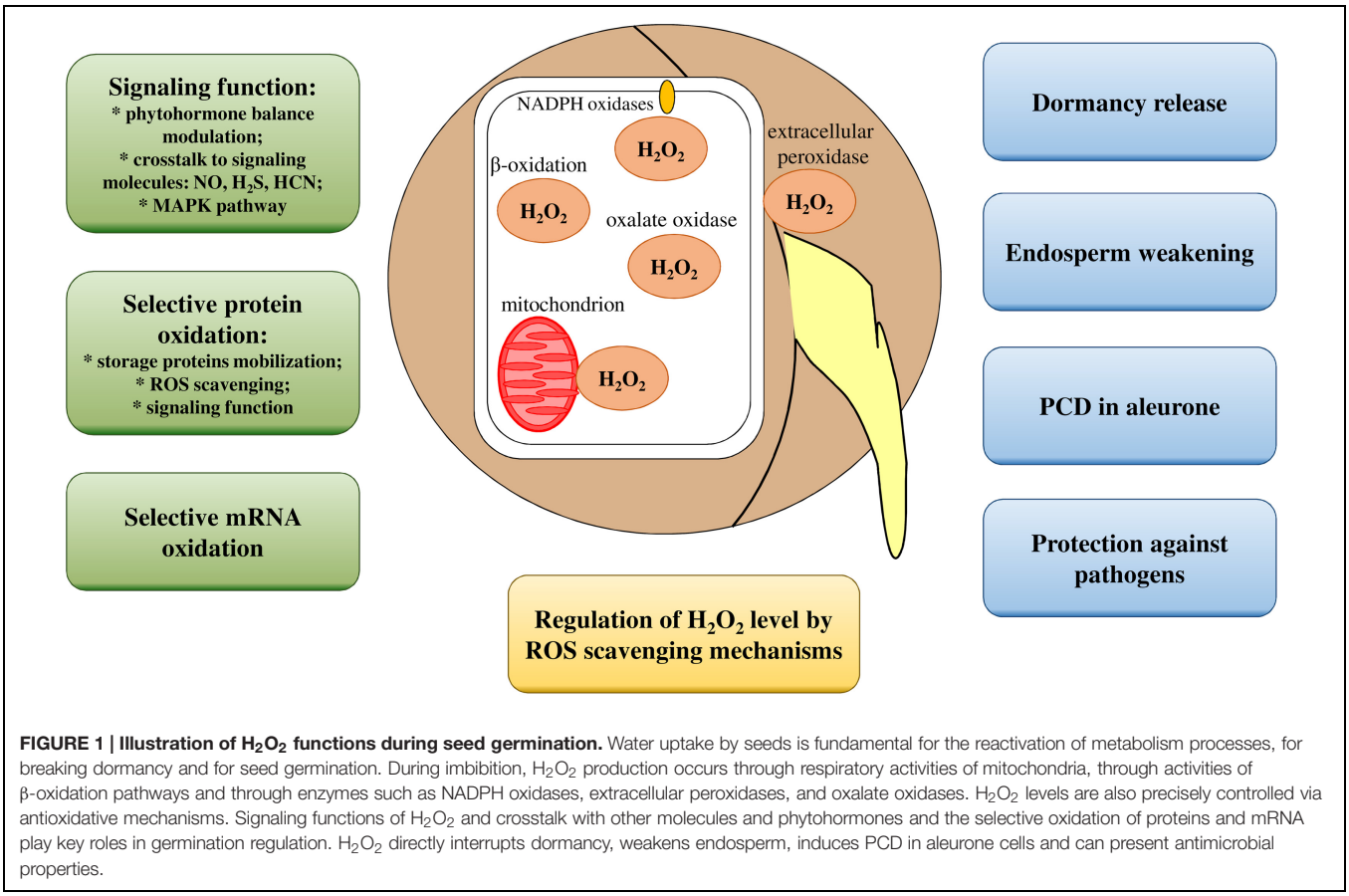

FIGURE 1 | Illustration of H_2O_2 functions during seed germination. Water uptake by seeds is fundamental for the reactivation of metabolism processes, for breaking dormancy and for seed germination. During imbibition, H_2O_2 production occurs through respiratory activities of mitochondria, through activities of β-oxidation pathways and through enzymes such as NADPH oxidases, extracellular peroxidases, and oxalate oxidases. H_2O_2 levels are also precisely controlled via antioxidative mechanisms. Signaling functions of H_2O_2 and crosstalk with other molecules and phytohormones and the selective oxidation of proteins and mRNA play key roles in germination regulation. H_2O_2 directly interrupts dormancy, weakens endosperm, induces PCD in aleurone cells and can present antimicrobial properties.

maturation can be essential to their future mobilization through proteolytic cleavage by the 20S proteasome, which facilitates their mobilization during germination and seedling establishment through the destabilization of a highly compact seed storage protein complex (Job et al., 2005).

Verma et al. (2015) postulated that H_2O_2 and ROS production during germination contribute to reserve mobilization through oxidative modifications of stored proteins, which may be recognized by storage organs as signals to mobilize reserves to the rapidly growing axis. Due to the high abundance of seed storage proteins available, their oxidized forms can also be treated as scavenging systems for ROS (Job et al., 2005; Barba-Espín et al., 2011). The oxidation of proteins such as glycolytic enzymes, mitochondrial ATP synthase, aldolase reductase, methionine synthase, translation factors, and molecular chaperones (seemingly treated as deleterious effects) is a positive stimulator of germination, as specific oxidation processes can help protect other cell components against the negative effects of ROS. Moreover, the impairment of some metabolic activities (e.g., glycolytic enzymes) may lead to the activation of the pentose phosphate pathway (PPP), providing reducing power for antioxidant enzymes in the form of NADPH (Job et al., 2005; Barba-Espín et al., 2011). Oracz et al. (2007) proposed a mechanism for seed dormancy release that involves a change in proteome oxidation resulting from the accumulation of ROS during after-ripening phase. As the breaking of dormancy, both in dry and imbibed seeds, is accompanied by ROS production and by the carbonylation of specific embryo proteins, they assume a more general version of this mechanism. Based on these data, it can be concluded that ROS play an important role in seed proteome and transcriptome remodeling by selective oxidation, which can trigger dormancy release and germination (Diaz-Vivancos et al., 2013).

The germination of *Arabidopsis*, black peppercorns (*Piper nigrum*) and tomatoes (*Lycopersicon esculentum*) is limited by a mechanical barrier (e.g., endosperm). Germination can proceed when the mechanical barrier in the endosperm decreases. ROS can participate in endosperm weakening during germination through cell wall loosening. Müller et al. (2006, 2007) showed that H_2O_2 abolishes inhibitory effects of abscisic acid (ABA) on endosperm rupture. It has also been shown that during lettuce (*Lactuca sativa*) seed germination, exogenous ROS and ROS generation inducers increase the percentage of endosperm cap ruptures (Zhang et al., 2014b). Lariguet et al. (2013) suggested that H_2O_2 regulates the expression of gene encoding enzyme hydrolyzing the testa and endosperm, which facilitate *Arabidopsis* germination by releasing the embryo from the control of the seed envelope. However, seed dormancy and germination is not only controlled by the transcriptional regulation of gene expression. Rather, it is also controlled through the management of mRNA abundance and protein functioning (El-Maarouf-Bouteau et al., 2015).

H_2O_2 likely regulates gene expression through protein oxidation, activation, and regulation of kinase transduction cascades, changes in the redox state of cysteine residues of transcription factors that regulate their activity and alteration in the cellular redox state, which is managed by ROS-antioxidant interactions (Job et al., 2005; Oracz et al., 2007; Barba-Espín et al., 2011; Bazin et al., 2011; Bykova et al., 2011a,b; El-Maarouf-Bouteau et al., 2013; Lariguet et al., 2013). Coordinate regulation at transcriptome and proteome levels during germination involves H_2O_2- and ABA-mediated signaling through the mitogen-activated protein kinases (MAPK) pathway (Barba-Espín et al., 2011) and through the receptor for activated C kinase 1 (RACK1; Zhang et al., 2014a). RACK1 is a member of the tryptophan-aspartate repeat family of proteins, which performs multiple signaling functions in the growth and development of all eukaryotes (including plants; Zhang et al., 2014a).

During germination, H_2O_2 also protects against pathogens. $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$ production in radish (*Raphanus sativus*) seeds has been shown to be a sign of the presence of active and developmentally controlled physiological processes that play a presumption role in protecting emerging seedlings from damages by pathogens (Schopfer et al., 2001). This hypothesis is based on the well-documented role of oxidative burst during pathogen infection, which leads to the induction of programmed cell death (PCD). However, ROS (mainly H_2O_2) also possess antimicrobial properties (Coll et al., 2011). Moreover, oxalate oxidase, which has previously been described as a germin, has been shown to catalyze the direct conversion of oxalate secreted by pathogenic fungi to CO_2 and H_2O_2 during the germination of numerous species (Bolwell and Wojtaszek, 1997). Some evidence that reveals that the role of H_2O_2 in protecting against pathogens during germination and early seedling development is derived from studies on isolated lupine (*Lupinus luteus*) embryonic axes inoculated with *Fusarium oxysporum*, which causes the accumulation of H_2O_2 and free radicals (Morkunas et al., 2004). Biotic interactions between germinating seeds and microorganisms can also influence ROS levels through the stimulation of antioxidative capabilities, as is the case when tomato seeds are treated with the endophytic plant symbiont *Trichoderma harzianum* (Mastouri et al., 2010). The positive effects of H_2O_2 on germination have also been described for cereal grains in reference to their roles in PCD in aleurone (Fath et al., 2002). However, recent studies have shown that H_2O_2 may also be involved in mechanisms of ROS-dependent α-amylase release in barley (*Hordeum vulgare*) aleurone cells (Ishibashi et al., 2012). A summary of processes that involve increased levels of H_2O_2 during germination is shown in **Figure 1**.

H_2O_2 CROSSTALK WITH PHYTOHORMONES

It is now widely accepted that H_2O_2 plays a dual function in living organisms during numerous metabolic processes under both neutral and stress conditions. H_2O_2 and other ROS can be generated as mechanisms that regulate plant growth, development, and responses to environmental stress through crosstalk with phytohormones. Recently published data support the existence of interactions between ROS and phytohormone signaling networks that modulate gene expression and cellular redox status (Xia et al., 2015b). Interrelationships and balance between phytohormones is of critical importance to the regulation of seed dormancy and germination (**Figure 2**) and has been reviewed and summarized in numerous studies (Brady and McCourt, 2003; Kucera et al., 2005; Finch-Savage and Leubner-Metzger, 2006; Daszkowska-Golec, 2011).

H_2O_2 interactions with phytohormones in the regulation of seed dormancy and germination are still a subject of discussion. Depending on the organs and biological processes involved, interactions between phytohormones and H_2O_2 can be either antagonistic or synergistic. ABA and gibberellic acid (GA) play opposite functions, and their roles in dormancy release and germination are essential and well established (Finch-Savage and Leubner-Metzger, 2006; Finkelstein et al., 2008; Nambara et al., 2010; Nonogaki et al., 2010; Weitbrecht et al., 2011; Graeber et al., 2012; Rajjou et al., 2012; Golldack et al., 2013), while the functions and interactions of other phytohormones during germination remain a subject of further research (Matilla and Matilla-Vázquez, 2008; Linkies et al., 2009; Linkies and Leubner-Metzger, 2012; Corbineau et al., 2014; Miransari and Smith, 2014). Pigeon pea (*Cajanus cajan*) seeds primed with auxin, cytokinin, GA, and ethylene (ET) exhibit improved germination results under both control and Cd-stress conditions (Sneideris et al., 2015). Similar effects have been observed during seed priming with ABA, but only at low concentrations. However, it is difficult to distinguish between positive effects of hydropriming and priming with phytohormones upon germination, as only high doses of ABA do not stimulate seed germination. This suggests that ABA inhibits germination at high concentrations, which has not been observed for auxin, cytokinin, GA, and ET (Sneideris et al., 2015).

The phytohormone cytokinin has been proposed to promote seed germination by antagonizing ABA suppression of seed germination. Interactions between ABA and cytokinin during seed germination and seedling growth mediated by interplays between transcriptional regulators have been found in *Arabidopsis* (Wang et al., 2011). The roles of interactions between cytokinin, ABA and GA in the regulation of heteromorphic plant dormancy and germination have been revealed through studies on *Suaeda acuminata* seeds (Wang et al., 2012b). Guan et al. (2014) identified a genetic pathway through which cytokinin specifically induces the degradation of ABI5 protein, thereby antagonizing the ABA-mediated inhibition of post-germinative growth in *Arabidopsis*. Functions of cytokinins in germination stimulation that involve counteracting inhibitory effects of ABA have been found in brown seed morphs of *Suaeda acuminata* (Wang et al., 2012b). Crosstalk between ABA and auxin has been found in numerous species, and auxin is known to affect germination in the presence of ABA, although the molecular mechanisms of such interactions remain unknown. Potential functions of ARF transcription factors and their regulation by miR160 in interactions between ABA and auxin during *Arabidopsis* seed germination and early growth were evidenced by Liu et al. (2007). Signaling processes trigger interactions

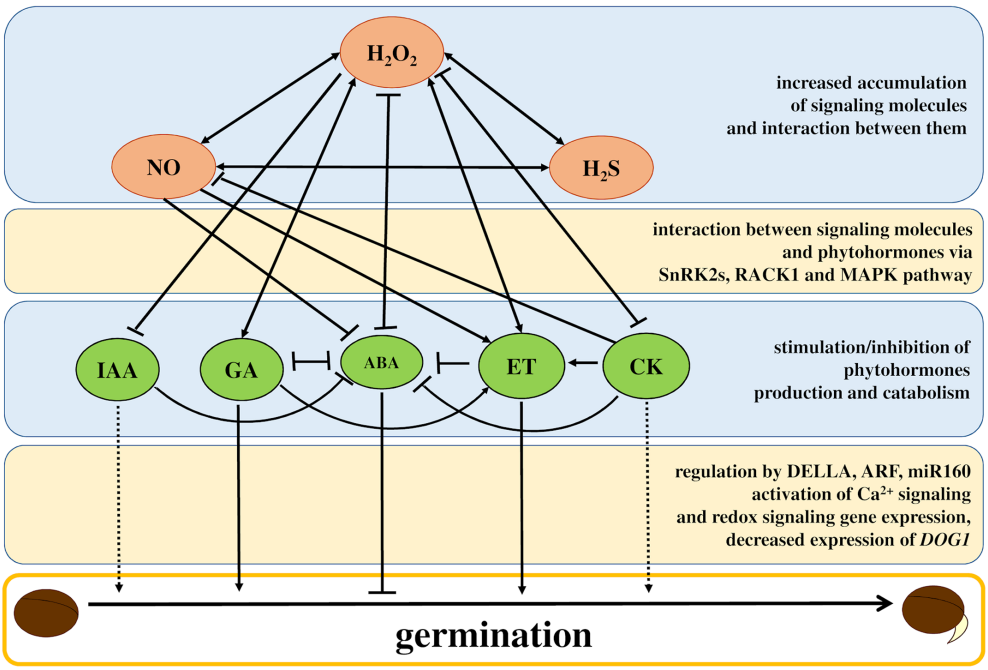

FIGURE 2 | Schema showing crosstalk between H_2O_2, NO, and H_2S and associated interactions with phytohormones for seed germination control. The accumulation of signaling molecules during imbibition influences phytohormone balance by decreasing abscisic acid (ABA), cytokinin (CK), and auxin (IAA) levels while increasing gibberellic acid (GA) and ethylene (ET). Combined effects of signaling molecules and phytohormones and signal transduction via SnRK2s, RACK1, and MAPK pathways lead to the control and regulation of transitions from the dormancy state to germination. Current concepts emphasize a primary role of GA and ET in promoting seed germination and germination inhibition by ABA while the function of IAA and CK in germination *sensu stricto* is debatable (marked by dashed line), however, both IAA and CK regulate cell division and cell growth in subsequent phases. The regulation of DELLA, ARF, miR160, and DOG gene expression and activity play key roles in this process. The phytohormonal regulation of dormancy breaking is accompanied by the activation of calcium signaling and redox signaling gene expression. Interactions and crosstalk between signaling molecules and phytohormones and their role in regulating germination are discussed in greater detail in the text. Positive or negative interactions are shown by arrows or bars, respectively.

not only between particular phytohormones but also between phytohormones and other signaling molecules such as NO (Arc et al., 2013b; Krasuska et al., 2015; Sanz et al., 2015), HCN (Oracz et al., 2008), H_2S (Jin and Pei, 2015), $^{\cdot}OH$ (Richards et al., 2015), and H_2O_2 (Diaz-Vivancos et al., 2013), which is believed to play a central role in signaling processes during plant development and stress responses (Petrov and Van Breusegem, 2012).

The seed germination of warm-season grasses is significantly responsive to oxidative conditions, and the complex interplay between seed redox status, ABA, H_2O_2, and NO in this system has been highlighted (Sarath et al., 2007a,b). Studies on phytohormone interactions in germinated seeds have shown that exogenously applied ABA inhibits ROS accumulation in barley (Ishibashi et al., 2012), rice (*Oryza sativa*; Ye et al., 2012), lettuce (Zhang et al., 2014b), and sunflower (El-Maarouf-Bouteau et al., 2015). By contrast, the addition of GA enhances the production of ROS, and mainly through superoxide and H_2O_2 found in radish plants (Schopfer et al., 2001) and *Arabidopsis* (Liu et al., 2010; Lariguet et al., 2013). Bahin et al. (2011) suggested that H_2O_2 plays a role in the alleviation of barley seed dormancy through the activation of GA signaling and/or biosynthesis rather than through the inhibition of ABA signaling. They found that exogenously applied H_2O_2 does not influence ABA biosynthesis and signaling but that it has a more pronounced effect on GA signaling, resulting in the modulation of hormonal balance and in subsequent germination initiation. The modulation of phytohormone balance during germination by exogenously applied H_2O_2 is likewise a product of changes in H_2O_2 levels in seeds treated with GA and ABA. Enhanced superoxide and H_2O_2 production has been observed in *Arabidopsis* seeds treated with GA, and declines in ROS have been found in seeds treated with ABA (Lariguet et al., 2013).

Studies on H_2O_2 exogenously supplied under different light conditions have shown that H_2O_2 can either promote or repress germination depending on light qualities present (Lariguet et al., 2013). These authors concluded that the H_2O_2-dependent promotion of germination depends on phytochrome but not on cryptochrome signaling, which requires the presence of ROS interactions with GA. SnRK2 (plant-specific serine/threonine kinases) are involved in plant responses to abiotic stress and in ABA-dependent plant development (Kulik et al., 2011). Nakashima et al. (2009) stated that SnRK2 protein kinases are essential to the control of *Arabidopsis* seed development and dormancy. Ishibashi et al. (2012) speculated that the relationship between SnRK2 and ROS constitutes an essential factor in seed germination and dormancy and proposed a model describing the interactions of ROS in GA and ABA signaling in barley aleurone

cells. Zhang et al. (2014a) concluded that *OsRACK1A* positively regulates rice seed germination by controlling endogenous levels of ABA and ROS and their interactions. *In silico* analysis suggests the presence of possible interactions between SnRK2 and RACK1, which may participate in signal transduction pathways that regulate seed dormancy and germination (Szklarczyk et al., 2015).

El-Maarouf-Bouteau et al. (2015) stated that ROS act together with ABA at the transcriptional level in sunflower plants mainly by decreasing the number of key targeted transcripts not through the stimulation of phytohormone-related gene expression required for germination (e.g., genes of GA or ET signaling pathways), but instead, through the set of genes related to calcium and redox signaling. They also suggest that the transcriptional regulation of sunflower seed germination is more closely related to the suppression of inhibitors than to the active transcription of stimulators. Barba-Espín et al. (2010) proposed an interaction between the redox state and phytohormones coordinated by H_2O_2 in the induction of proteins associated with plant signaling and development during pea seed germination. They observed better germination performance accompanied by decreases in ABA, zeatin-riboside, salicylic acid, jasmonic acid, and indole acetic acid levels in germinated peas with exogenously supplied H_2O_2, supporting their conclusion that H_2O_2 can directly act as a messenger within the phytohormonal network and as a signaling molecule involved in the germination of orthodox seeds. The central and integrative role of H_2O_2 in the regulation of sunflower seed germination via phytohormones such as ET, ABA, GA, jasmonic acid, and salicylic acid was also postulated by El-Maarouf-Bouteau et al. (2015).

The function of H_2O_2 as a management center that balances phytohormone interactions for germination purposes could occur via MAPK (Barba-Espín et al., 2011). Two mechanisms for H_2O_2-driven MAPK signaling in germinating pea seeds have been proposed. According to the first model, exogenously added H_2O_2 induces a MAPK-dependent decrease in ABA content in seeds. The second model assumes direct or indirect negative effects of H_2O_2 on ABA transport from the cotyledon to the embryonic axis, resulting in a decrease in ABA. Finally, decreases in ABA may induce a MAPK-mediated reduction in the ET precursor (ACC, 1-aminocyclopropane carboxylic acid), favoring germination (Barba-Espín et al., 2011, 2012). One study on the interactive roles of GA, ABA and ET and on the possible involvement of ROS in the mediation of phytohormone actions during mung bean (*Vigna radiata*) seed germination shows that ET essentially has a positive effect on seed germination with possible interactions with ROS (Chaudhuri et al., 2013).

Ethylene may mainly promote radial cell expansion in the embryonic hypocotyl, increase seed respiration, decrease seed base water potential, or enhance the expression of cell wall hydrolases in the endosperm cap (Chaudhuri et al., 2013). Linkies et al. (2009) showed that the inhibitory effects of ABA on *Lepidium sativum* seed germination are counteracted by ET and proposed a model on the phytohormonal regulation of endosperm cap weakening and rupture. Observations on germinating lettuce seeds show that when seeds are imbibed in water, the H_2O_2 content in the cap increases prior to cap rupture and decreases thereafter, whereas H_2O_2 content in the radicle remains very low (Zhang et al., 2014b). El-Maarouf-Bouteau et al. (2015) proposed that ET production at the end of the pea seed germination process correlates with ROS accumulation and that ROS and ET together participate in the initiation of cell elongation (the first visible symptom of germination completion), which has also been suggested for apples (*Malus domestica*; Gniazdowska et al., 2010a,b) and soybeans (*Glycine max*; Ishibashi et al., 2013) and in reference to the initiation of cell division.

Corbineau et al. (2014) proposed that ET plays a central role in seed dormancy regulation via crosstalk between phytohormones and other signals, although information on the interrelationship between ET and H_2O_2 in the regulation of seed germination remains limited and inconsistent. Various mechanisms that fine-tune ROS production and accumulation operate during seed germination (and include the action of phytohormones). Antioxidant functions of cytokinin in healthy soybean seeds have been postulated by Gidrol et al. (1994). The accumulation of ROS during germination leads to the oxidation of endogenous cytokinin (Gidrol et al., 1994), which abolishes their functions. Cytokinins also interact with NO, thus demonstrating that antagonistic effects on seed germination and can act as suppressors of NO, as shown for *Arabidopsis* (Liu et al., 2013).

H_2O_2 CROSSTALK WITH SIGNALING MOLECULES

While the role of H_2O_2 and NO in seed biology has been studied widely, knowledge regarding the functions of other molecules and on their interactions remains scarce. Both NO and H_2O_2 perform a parallel function in terms of interrupting germination dormancy and stimulation through interactions with ABA (**Figure 2**). In reference to seed physiology, the model on crosstalk between ROS, NO, and ABA differs from the well-established model on stomatal guard cell regulation (Arc et al., 2013a,b). Seed imbibition increases H_2O_2 and NO levels. H_2O_2 up-regulates ABA catabolism (most likely through an NO signal) while also promoting GA biosynthesis (Liu et al., 2010; Arc et al., 2013b). Similar to H_2O_2, the exogenous application of NO imposes seed dormancy and diminishes the inhibitory effects of ABA on seed germination (Bethke et al., 2004, 2006). The application of NO also stimulates seed germination under stress conditions (Kopyra and Gwóźdź, 2003; Zheng et al., 2009).

Liu et al. (2010) proposed a hypothetical model that explains interrelationships between H_2O_2 and NO in the regulation of seed germination by joint actions of ABA and GA. According to this model, H_2O_2 can interrupt the dormancy of *Arabidopsis* seeds through two pathways. The first pathway relies on the enhancement of ABA catabolism and GA biosynthesis. The signaling molecule (NO) does not regulate GA biosynthesis directly but instead acts as a temporary signaling molecule involved in the H_2O_2 regulation of ABA catabolism. The second

pathway assumes the negative regulation of GA biosynthesis by ABA. Bahin et al. (2011) suggested that H_2O_2 interrupts dormancy in barley seeds through GA signaling activation rather than influencing ABA metabolism. Gniazdowska et al. (2010c) proposed a function for H_2O_2 in apple seed germination and its role in the downstream signaling of NO and HCN in the activation of ET biosynthesis during early seedling growth. They also found that the activities of crucial enzymes involved in ET metabolism are modified by HCN and NO treatments.

Oracz et al. (2009) presented a comprehensive scheme on the mechanism of HCN-dependent dormancy alleviation and on its crosstalk to ROS as a decisive signaling element involved in seed germination. The dominant role of ROS and reactive nitrogen species (RNS) in the regulation of seed dormancy and germination is also discussed. The authors postulate that NO may play a key role in germination vigor, which may result from crosstalk between NO and ROS (Arc et al., 2013a). Krasuska and Gniazdowska (2012) stated that ROS, NO, and HCN can simultaneously affect embryo dormancy release processes and that their accurate levels are essential to seed germination and development regulation. Based on the "oxidative window" model, a model proposed by Bailly et al. (2008) that describes regulating functions of ROS in seed dormancy/germination switch, Krasuska and Gniazdowska (2012) presented the "nitrosative door" hypothesis, which focuses on the concentration-dependent role of RNS (mainly in terms of NO in seed physiology). They also proposed that RNS and ROS levels are strictly regulated by ROS scavenging enzymes.

Wang et al. (2015b) presented a mechanism of NO suppression on the inhibitory effects of ABA on seed germination. Based on studies related to interactions between ABA, NO, and ROS in stomatal guard cells and based on their own results, Wang et al. (2015b) suggested that NO negatively regulates ABA signaling through *S*-nitrosylation of SnRK2s proteins (SnRK2.2, SnRK2.3, and SnRK2.6/OST1) not only in terms of stomatal closure but also in terms of the inhibition of seed germination and seedling growth. They proposed that *S*-nitrosylation of SnRK2s proteins serves as a key component of signaling crosstalk between ABA and NO that regulates *Arabidopsis* seed germination. They described a mechanism for NO involvement in dormancy release and germination promotion. Based on their findings, endogenous and exogenously applied NO exerts inhibitory effects on the kinase activities of SnRK2.2 and SnRK2.3 via *S*-nitrosylation and thus blocks ABA signaling (Wang et al., 2015b).

Interest in the H_2S molecule has grown in plant biology research. This is due to its signaling functions and interactions with H_2O_2 and NO during plant development and stress responses (Calderwood and Kopriva, 2014; Hancock and Whiteman, 2014, 2015; Jin and Pei, 2015) and during seed germination (Li, 2013). Improved germination and decreases in germination time periods have been observed in common bean (*Phaseolus vulgaris*), maize (*Zea mays*), wheat (*Triticum aestivum*), and pea seeds subjected to H_2S treatments (Dooley et al., 2013). These results suggest that H_2S plays an important role as a signaling molecule that can accelerate growth rates of numerous plant species. Positive effects of H_2S and H_2O_2 treatments on the promotion of mung bean seed germination have been observed by Li and He (2015). These authors suggest that H_2O_2 and H_2S may promote the germination of mung bean seeds by mobilizing reserve proteins and that H_2O_2 may serve as a downstream signaling molecule of H_2S.

Li et al. (2012) proposed the existence of crosstalk between H_2O_2 and H_2S during seed germination. The authors found improved germination percentages for *Jatropha curcas* seeds soaked in H_2O_2 accompanied by an increase in L-cysteine desulfhydrase activity that induce H_2S accumulation. Moreover, Li et al. (2012) observed better germination performance after adding H_2S to a soaking solution and postulated that this improvement is mediated by H_2S. In the signaling process mediated by H_2S during seed germination, both H_2O_2 and NO play important roles. In NaCl-stressed alfalfa (*Medicago sativa*) seeds, both H_2S (sodium hydrosulfide) and NO donors (sodium nitroprusside) can significantly attenuate seed germination and seedling growth inhibition by protecting against oxidative damage (Wang et al., 2012a). The authors also showed that the application of H_2S donor enhances NO accumulation while the addition of 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), a specific NO scavenger, diminishes positive impacts of H_2S on germination and NaCl stress tolerance, suggesting the presence of interactions between H_2S and NO in germinating seeds. A schematic illustration of interrelationships and crosstalk between signaling molecules and phytohormones during the regulation of seed germination is presented in **Figure 2**.

H_2O_2 PRIMING-INDUCED ABIOTIC STRESS TOLERANCE

Seed vigor is an important agronomic trait that determines a seed's potential for rapid uniform emergence and development under a broad range of field conditions (Catusse et al., 2008; Rajjou et al., 2012; Ventura et al., 2012). Modern approaches to seed quality improvement involve classical genetics, molecular biology and invigoration treatments known as priming treatments. Seed priming is a pre-sowing treatment that is widely used in the vegetable and flower seed industry to enhance seedling establishment, crop stands and yields (Bradford, 1986; Di Girolamo and Barbanti, 2012; Bewley et al., 2013; Jisha et al., 2013; Paparella et al., 2015). This technique involves imbibing seeds with restricted amounts of water to create hydration conditions that permit pre-germinative metabolic events to proceed while preventing radicle protrusion.

In most plant species, seeds remain desiccation-tolerant prior to radicle emergence (Hilhorst et al., 2010), and thus seeds can be dried to their original moisture levels after being soaked for storage, distribution and sowing via conventional techniques. However, primed seed storage is a major challenge involved in seed priming (Argerich et al., 1989; Hussain et al., 2015). Priming treatments have beneficial effects on seed vigor and viability, which manifest as improved germination performance (increased germination rates, total germination percentages, and germination uniformity) and plant growth,

especially under adverse environmental conditions (**Figure 3**), (Ashraf and Foolad, 2005; Chen and Arora, 2011; Yacoubi et al., 2011; Chen et al., 2012; Jisha and Puthur, 2015; Kubala et al., 2015a,b; Salah et al., 2015). Depending on the plant species, seed morphology and physiology, a variety of physicochemical and biological priming treatments can be applied. Currently available priming techniques include hydropriming (soaking seeds in predetermined amounts of water or limiting imbibition periods), osmopriming [soaking seeds in osmotic solutions (e.g., PEG) or in salt solutions], matrix priming (mixing seeds with organic or inorganic solid materials and water in known proportions and in some cases adding chemical or biological agents), chemical priming (soaking seeds in various chemical solutions), hormonal priming (treating seeds with plant growth regulators) and biological priming/biopriming (using beneficial microorganisms to seed during priming; Di Girolamo and Barbanti, 2012; Jisha et al., 2013; Paparella et al., 2015).

Priming enhanced germination performance may be considered a result of advanced germination metabolism processes (Soeda et al., 2005) involving more efficient respiratory pathways (Li et al., 2010; Sun et al., 2011), enhanced antioxidant activity (Bailly et al., 1998, 2000; Posmyk et al., 2001; Chen and Arora, 2011; Yacoubi et al., 2011), initiated repairing processes (Balestrazzi et al., 2011; Kibinza et al., 2011), and altered phytohormonal balance (El-Araby et al., 2006). Higher expressions of genes and proteins involved in water transport, cell wall modification, cytoskeletal organization, and cell division and increases in protein synthesis potential, post-translational processing capacity, and targeted proteolysis have been linked to the advanced germination of primed seeds (Gao et al., 1999; Gallardo et al., 2001; Chen and Arora, 2013; Kubala et al., 2015a). However, priming involves other specific mechanisms that improve germination and thus priming cannot simply be viewed as an acceleration of germination-related processes.

Priming procedures can generate moderate levels of abiotic stress during both soaking (e.g., osmotic stress, salinity, and drought generated by priming agents) and dehydration phases (Ashraf and Foolad, 2005; Kubala et al., 2015a). This abiotic stress generated during priming can activate stress-responsive systems in primed seeds that lead to enhanced tolerance levels to subsequent stress during post-priming germination and seedling establishment (Chen and Arora, 2013). Beneficial effects of seed priming are also observed during more advanced developmental stages (Bruce et al., 2007; Patade et al., 2011) and throughout the entire growing season (Iqbal and Ashraf, 2006, 2007; Hussain et al., 2014). It will be worthwhile to determine whether seed priming effects can be perpetuated to the next generation as in the case of priming-induced transgenerational resistance that protects future generations against biotic stress (Luna and Ton, 2012; Slaughter et al., 2012).

Seed priming improves the stress tolerance of germinating seeds and plants grown from primed seeds based facilitating faster transitions to the germinated state and the activation of stress-responsive systems. These two strategies constitute "priming memory" or stress imprinting mechanisms, which cover genetic or biochemical modifications induced by priming, which in turn can occur as a result of subsequent stress exposure and which mediate enhanced tolerance of subsequent stress (Chen and Arora, 2013). Kubala et al. (2015b) showed that after initial stress exposure, primed rape (*Brassica napus*) seeds present salinity stress tolerance during post-priming germination, a feature that is likely linked to "priming memory."

In addition to water-based priming with controlled seed imbibition, priming treatments on vegetative plant parts are often used. Initial plant exposure to stressors or chemical compounds results in the faster and stronger induction of basal resistance mechanisms upon the onset of subsequent pathogen attacks or in greater tolerance against abiotic stresses, as reviewed by Tanou et al. (2012), Pastor et al. (2013), and Hossain et al. (2015). Numerous priming-inducing chemicals are endogenous plant compounds (e.g., H_2O_2). Beneficial effects of exogenous H_2O_2 applied in the form of foliar spray on the induction of tolerance to drought, salinity, chilling, high temperatures, and heavy metal stress, all of which modulate H_2O_2 production, were recently summarized by Hossain et al. (2015). These authors proposed a hypothetical model on the effects of H_2O_2 on plant defense mechanisms associated with abiotic stress. It is speculated that H_2O_2 treatments that involve seed soaking or the use of foliar spray induce low levels of oxidative stress and that ROS (especially H_2O_2-dependent signaling networks) induce the accumulation of latent defense proteins, resulting in the generation of primed states and in improved stress responses (Hossain et al., 2015). Positive effects of seed priming with H_2O_2 in terms of enhancing salt and high temperature tolerance in barley, drought tolerance in wheat and common bean and soil moisture stress tolerance in cotton (*Gossypium hirsutum*) have been shown (He et al., 2009; Cavusoglu and Kabar, 2010; Abass and Mohamed, 2011; Santhy et al., 2014). Priming with H_2O_2 also improves spring maize seedling growth under suboptimal temperatures (Ahmad et al., 2015).

The above findings show that the stress tolerance of germinating seeds and seedlings brought about through seed pretreatment with H_2O_2 is attributable to the reduction of endogenous H_2O_2 content, to more effective antioxidative systems, to the expression of stress proteins, to improved membrane stability and to high photosynthetic capacity, which help alleviate damage while stimulating growth under stress (**Figure 3**). Enhanced levels of endogenous H_2O_2 accompanied by proline accumulation have been observed during the osmopriming and post-priming germination of rape seeds (Kubala et al., 2015b). The authors have stated that higher levels of H_2O_2 accumulation in primed seeds associated with higher proline content, gene expression, and enzymatic activity of pyrroline-5-carboxylate synthetase (P5CS) suggest that H_2O_2 and proline play a crucial role in improving salinity tolerance by osmopriming. However, tomato priming with various priming solutions (kinetin, KNO_3, NaCl, KH_2PO_4, and $CaCl_2{\cdot}2H_2O$) were decreases the H_2O_2 production under NaCl stress (Theerakulpisut et al., 2011).

The treatment of seeds with exogenous H_2O_2 and subsequent germination without drying to initial moisture content (MC) improves the salt tolerance of wheat seedlings by alleviating oxidative damage, enhancing stress protein expression (Wahid et al., 2007), aluminum-induced oxidative stress mitigation in

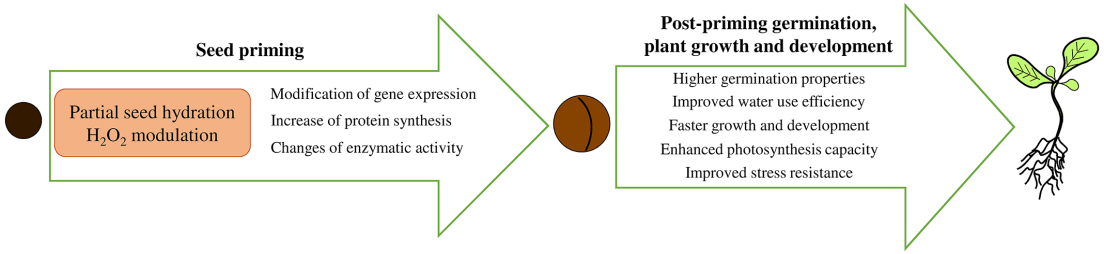

FIGURE 3 | Schema on the central role of priming-induced hydrogen peroxide modulation in improving post-priming germination, seedling growth, and stress tolerance. Partial seed hydration during pre-sowing priming and H_2O_2 metabolism modulation control cellular processes through gene expression regulation, increased protein synthesis, and changes in enzymatic activity.

wheat seedlings (Xu et al., 2011a) and antioxidant system and nutrient relation modulation in maize plants under water-deficit conditions (Ashraf et al., 2015). Seed treatment with H_2O_2 also enhances the germination and seedling growth of sunflower plants and modifies net photosynthetic rates and antioxidant systems in mung bean plants under non-stressed conditions (Wahid et al., 2008; Khan et al., 2015). Comparisons between the effectiveness of the surface drying and re-drying of soaked seeds show no difference between these two strategies in regards to post-priming germination and seedling growth (Farooq et al., 2010). In taking these results into account, it can be concluded that the pretreatment of seeds with H_2O_2 may have similar effects on germination performance, seedling growth, and metabolic processes as priming treatments, although it seems that dehydration after seed soaking plays an important role in the regulation of gene expression and protein accumulation (Kubala et al., 2015a).

Regardless of the seed invigoration method applied, enhanced abiotic stress tolerance was achieved through H_2O_2 level modulation and regulation of multiple stress-responsive pathways. The capacity to alleviate the production of ROS serves as an important component of stress tolerance in both seeds and plants (Kranner and Seal, 2013). Seed heteromorphism, i.e., the production of different seed morphs with different germination characteristics by a single individual, has been found in a number of halophytic taxa as a means of responding to harsh environments (Li et al., 2005; Cao et al., 2015). Studies on seedlings derived from dimorphic seeds of *Atriplex centralasiatica* reveal differential salt tolerance levels as a result of different levels of H_2O_2 caused by the modulation of antioxidative enzyme activities by NO (Xu et al., 2011b).

It is well established that primed seeds are developmentally more advanced to reach complete germination than unprimed ones (Chen and Arora, 2013). Similarly, treating seeds with activators of plant defense against pathogen and herbivores is not accompanied by a reduction in growth (Worrall et al., 2012). Plant priming for the enhanced induction of defense responses is often accompanied by compromised plant performance (Chinnusamy and Zhu, 2009) but requires lower fitness costs than the direct induction of defenses (Van Hulten et al., 2006). Seed priming is economically more attractive than chemical plant treatments applied to plants in field conditions. Therefore, a further examination of molecular mechanisms that support seed priming is not only of fundamental importance but also of practical importance, as such studies may help us to uncover fruitful agricultural strategies.

ROLES OF H_2O_2 IN SEED AGING

Seed aging involves the gradual accumulation of damage to cellular components, which in turn results in a loss of seed viability and vigor. This process occurs during prolonged seed storage and escalates when seeds are stored in improper conditions (especially in high temperature and moisture conditions; Arc et al., 2011). As stored seeds lose longevity over time, it is critically important to understand the mechanisms of the aging process that are related to agronomic and ecological (*ex situ* seed conservation) factors. It is worth emphasizing that rates of aging and seed longevity vary between species. The lifespan of stored seeds depends not only on storage periods and conditions but also on genetic, physiological, and morphological factors (seed structures, compositions of reserves, seed maturation programs, etc.; Walters et al., 2005; Ventura et al., 2012). Deleterious effects of seed aging are commonly examined using artificial aging methods (controlled deterioration test, CDT and accelerated aging, AA) that involve seed exposition to high temperature ($\geq$35°C) and humidity ($\geq$75%RH) conditions for relatively short periods of time (Black et al., 2006). These techniques are designed to hasten and mimic the natural aging process (the prolonged storage of dry seeds). However, doubts have been raised regarding whether CDT and AA treatments accurately convey mechanisms of seed deterioration as a result of natural aging processes (Murthy et al., 2003; Lehner et al., 2008; Schwember and Bradford, 2010; Groot et al., 2012). Differences may result mainly from the partial hydration of seeds during CDT and AA, which can activate biochemical pathways not found in dry seeds (Bewley et al., 2013).

Although the biochemical and molecular basis of the seed aging process is still not fully understood, it is well established that seed aging causes several deleterious changes within cells (e.g., DNA damage, a loss of RNA synthesis reflecting impaired protein production, a loss of membrane integrity, mitochondrial dysfunction, protein inactivation, telomere shortening, etc.;

McDonald, 1999; Fu et al., 2015). According to the "free radical theory of aging," the driving force behind most alterations that occur during the aging of living organisms is ROS activity. This assumption also refers to aged seeds and is supported by numerous reports (Rajjou et al., 2008; Bellani et al., 2012; Hu et al., 2012; Parkhey et al., 2012; Yao et al., 2012; Xin et al., 2014; Ratajczak et al., 2015). ROS production in dry stored seeds ensues as a result of non-enzymatic processes (e.g., Amadori and Maillard reactions and lipid peroxidation; El-Maarouf-Bouteau and Bailly, 2008).

Oxidative damage in dry seeds may also be propagated as a result of inefficient enzymatic antioxidant machinery operating under low water content conditions. When seeds are hydrated to a certain extent (e.g., during artificial aging or seed storage in uncontrolled environments), ROS synthesis also occurs as a result of enzymatic reactions and respiratory activities (Bewley et al., 2013). H_2O_2, as a long-lived ROS, is able to migrate across membranes over relatively long distances and thus contribute to the aging process (Kibinza et al., 2006, 2011; Lehner et al., 2008; Xin et al., 2014; Kalemba et al., 2015; Kong et al., 2015; Ratajczak et al., 2015). Negative interrelationships between the viability/germination capacities of seeds and H_2O_2 accumulation during aging have been shown for artificially aged sunflower (Bailly et al., 1996; Kibinza et al., 2006, 2011), beech (*Fagus sylvatica*; Pukacka and Ratajczak, 2005, 2007), and wheat seeds (Lehner et al., 2008) and for naturally aged cotton (*Gossypium hirsutum*; Goel and Sheoran, 2003) and beech seeds (Ratajczak et al., 2015).

Kibinza et al. (2006) found that H_2O_2 levels in the embryonic axis depend on seed moisture levels and increase in a sublinear manner with increasing water content. Positive relationships have also been found between H_2O_2 production and energy metabolism, indicating that respiratory electron transport enhancement as a result of higher water status leads to the overproduction of H_2O_2, which in turn induces ATP depletion in aged seeds. Thus, MC seems to play a major role in seed deterioration (Kibinza et al., 2006). An analogous trend in terms of H_2O_2 level changes as a function of MC was obtained for artificially aged oat (*Avena sativa*), wheat, and beech seeds (Pukacka and Ratajczak, 2005; Lehner et al., 2008; Kong et al., 2015). However, in aged oat seeds, H_2O_2 accumulation is only associated with MC over long storage periods (Kong et al., 2015). An increasing amount of H_2O_2 and of other reactive oxygen species during seed deterioration is also a reflection of the progressive depletion of enzymatic scavenger activities. Alterations of activity and of transcript levels of key antioxidant enzymes have been observed in aged seeds of different species (Bailly et al., 1996; Goel et al., 2003; Kibinza et al., 2006, 2011; Pukacka and Ratajczak, 2007; Lehner et al., 2008; Yao et al., 2012; Chen et al., 2013; Morscher et al., 2015; Ratajczak et al., 2015; Xia et al., 2015a).

A study on oat seeds showed that enzymatic antioxidants such as CAT, APX, and SOD can protect against oxidative stress in stored seeds with low MC, whereas when high levels of MC are present, these enzymes are heavily limited, and proline seems to play a more prominent role in the response to oxidative stress (Kong et al., 2015). The effects of $O_2^{\bullet -}$ and H_2O_2 on seed viability during storage under different temperatures were examined in black poplar (*Populus nigra*). The authors showed that after 2 years of storage, H_2O_2 accumulation is responsible for alterations of membrane permeability as a result of the changing compositions of fatty acids and phospholipids (Kalemba et al., 2015).

In naturally aged beech seeds, the production of H_2O_2 and of other ROS ($O_2^{\bullet -}$, $^{\bullet}OH$) is significantly higher in the embryonic axis than in cotyledons, suggesting that embryonic axes are more sensitive to storage and damage (e.g., DNA fragmentation). Nevertheless, whether found in the embryonic axis or in cotyledons, ROS accumulation is dependent on seed storage periods and it is accompanied by a loss of membrane integrity. Based on results obtained via the *in situ* localization of H_2O_2, $O_2^{\bullet -}$, and $^{\bullet}OH$, the authors suggest that losses in germination ability may also be a result of ROS-derived deleterious effects on cell division processes in root apical meristems of stored seeds, thus leading to the prevention of radicle protrusion (Ratajczak et al., 2015). However, some published data call the main role of H_2O_2 and of other reactive species in the aging process into question (Cakmak et al., 2010; Yin et al., 2015). In naturally aged alfalfa seeds, lipid peroxidation is the main product of long-term storage, although there is no correlation with H_2O_2, as the latter remains at a low level in aged dry seeds (Cakmak et al., 2010).

More recently, Yin et al. (2015) found that artificial aging treatments delay rape seed germination and increase ion leakage but do not promote H_2O_2 generation or the accumulation of any antioxidant enzymes (apart from peroxiredoxin). However, CDT treatments were found to affect SOD and CAT activates. The authors suggest that in *Brassica napus,* the over-accumulation of ROS does not act as a primary factor in initiating seed deterioration and other mechanisms (e.g., germination inhibitor synthesis and ABA content enhancement) are involved in the aging process (Yin et al., 2015). Some data indicate that losses in seed viability during aging are related to PCD (Kranner et al., 2011; Chen et al., 2013). As H_2O_2 and other ROS are considered to act as main modulators that control PCD in plant tissues (Gadjev et al., 2008), these molecules are likely also involved in signal transduction mediation that leads to PCD in aged seeds. Kibinza et al. (2006) speculated that H_2O_2-dependent decreases in ATP may result in cytochrome *c* release and thus may evoke PCD and losses in aged seed viability.

Observations made by El-Maarouf-Bouteau et al. (2011) show that PCD is found in hydrated seed states during the aging process. They proposed a scenario in which ROS together with by-products of lipid peroxidation trigger PCD in artificially aged seeds via DNA damaging (DNA laddering) and impaired mitochondrial functions. Associations with ROS and PCD were also found through CDT treatments applied to elm seeds (*Ulmus pumila*; Hu et al., 2012; Wang et al., 2015a). Transcriptional studies on aged pea seeds show that during the aging process, PCD-related and antioxidant gene expression levels change, leading to the progression of PCD and to the reduction of antioxidant capacity, which in turn eventually contribute to a loss of seed viability (Chen et al., 2013). Nevertheless, the impairment of seed viability by ROS-initiated PCD during aging has not been fully elucidated and requires further examination.

Some reports have shown that seed priming contributes to the alleviation of deleterious effects of seed aging (Bailly et al., 1998; Chiu et al., 2002; Goel et al., 2003; Butler et al., 2009). Priming with water and ascorbic acid improves the germination percentage of artificially aged cotton seeds concomitant with the lowering of lipid peroxidation and the partial restoration of antioxidant enzyme activities (CAT, SOD, POD, and GR in particular; Goel et al., 2003). Kibinza et al. (2011) showed that osmopriming applied after the artificial aging of sunflower seeds improves germination percentages independent of the aging period. Similarly, osmopriming leads to a significant drop in H_2O_2 and to the reestablishment of both catalase activity and *CAT1* transcript content. Their analysis of *in situ* CAT localization showed that this enzyme is also found with H_2O_2 in the cytosolic area. The authors concluded that CAT is a pivotal enzyme that protects against damages caused by ROS activities in aged seeds subjected to priming treatments (Kibinza et al., 2011).

CONCLUSION AND PERSPECTIVES

Seeds are of fundamental importance to plants as a means of propagation, and thus germination constitutes a critical phase as seeds transition from dormant to metabolically active states through to growth commencement and further development. Seeds are also exceedingly important to humans due to their function as a major source of crop production. As seeds are evidently of great biological and economic importance, precise knowledge of combined environmental and endogenous signals that regulate germination capacities are of great importance. Numerous studies have been conducted on cultivated plants for agricultural and economic purposes and on model plants (mainly *Arabidopsis*) for understanding cellular, biochemical and molecular processes that affect dormancy and germination. Crosstalk between the H_2O_2 signaling pathway and other signaling molecules such as NO and H_2S and phytohormones such as ABA, GA, and ET play an integrative role in switches made between dormant and germinated states (**Figure 2**). The accumulation of H_2O_2 and of other ROS during storage facilitates germination and has deleterious effects on seed viability.

It has been shown that pre-sown seed priming can be applied to improve seed quality, resulting in better germination performance and higher vigor while partially abolishing seed aging effects. Priming also influences signaling pathways through interactions with H_2O_2 metabolism (**Figure 3**). The exact mechanisms and functions of H_2O_2 during the germination of primed seeds must be clarified. One avenue for future research will involve identifying seed priming effects on the modulation of H_2O_2-mediated signaling networks. The use of numerous mutants and the development of new techniques will generate new perspectives that facilitate the more comprehensive explanation and substantiation of reviewed processes.

AUTHOR CONTRIBUTIONS

All of the authors have substantially contributed to the conception of this work and have jointly participated in drafting the manuscript and in preparing the figures. All of the authors critically revised the content of this work for key intellectual content and approved of its submission for publication. All of the authors have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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REVIEW
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Hydrogen Peroxide Signaling in Plant Development and Abiotic Responses: Crosstalk with Nitric Oxide and Calcium

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Hydrogen peroxide (H_2O_2), as a reactive oxygen species, is widely generated in many biological systems. It has been considered as an important signaling molecule that mediates various physiological and biochemical processes in plants. Normal metabolism in plant cells results in H_2O_2 generation, from a variety of sources. Also, it is now clear that nitric oxide (NO) and calcium (Ca^{2+}) function as signaling molecules in plants. Both H_2O_2 and NO are involved in plant development and abiotic responses. A wide range of evidences suggest that NO could be generated under similar stress conditions and with similar kinetics as H_2O_2. The interplay between H_2O_2 and NO has important functional implications to modulate transduction processes in plants. Moreover, close interaction also exists between H_2O_2 and Ca^{2+} in response to development and abiotic stresses in plants. Cellular responses to H_2O_2 and Ca^{2+} signaling systems are complex. There is quite a bit of interaction between H_2O_2 and Ca^{2+} signaling in responses to several stimuli. This review aims to introduce these evidences in our understanding of the crosstalk among H_2O_2, NO, and Ca^{2+} signaling which regulates plant growth and development, and other cellular and physiological responses to abiotic stresses.

Keywords: hydrogen peroxide (H_2O_2), nitric oxide (NO), calcium (Ca^{2+}), signal molecule, crosstalk

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INTRODUCTION

Hydrogen peroxide (H_2O_2), a form of reactive oxygen species, is regarded as a common cellular metabolite. H_2O_2 is continually synthesized through various sources including enzyme and non-enzyme pathways in plants. To date, it has become accepted that H_2O_2 plays important roles in plant developmental and physiological processes including seed germination (Barba-Espín et al., 2011), programmed cell death (PCD; Cheng et al., 2015; Vavilala et al., 2015), senescence (Liao et al., 2012b), flowering (Liu et al., 2013), root system development (Liao et al., 2009; Ma et al., 2014; Hernández-Barrera et al., 2015), stomatal aperture regulation (Ge et al., 2015) and many others. It is now clear that H_2O_2 functions as a signaling molecule which may respond to various stimuli in plant cells. These results suggest that H_2O_2 may be involved in cellular signaling transduction pathways and gene expression modulations in plants.

Nitric oxide (NO), as a small signaling molecule, appears to be involved in plant developmental and physiological processes such as seed germination (Wang et al., 2015), ripening and senescence (Shi Y. et al., 2015) as well as stomatal closure (Shi K. et al., 2015) and pollen tube growth (Wang et al., 2009). Meanwhile, NO signaling may have a vital role in the disease resistance

(Kovacs et al., 2015) and response to abiotic stresses such as cold (Fan et al., 2015), salt (Liu W. et al., 2015) and drought (Shan et al., 2015). Calcium ion (Ca^{2+}) signaling is also a core regulator of plant physiological process and stress adaption such as cell polarity regulation (Zhou et al., 2014), leaf de-etiolation (Huang et al., 2012), stomatal closure (Zou et al., 2015). Additionally, Ca^{2+} signaling is also involved in various responses to abiotic stimuli, including light (Hu et al., 2015) and heavy metal (Li et al., 2016).

A large amount of research show that H_2O_2, NO and Ca^{2+} as signaling are involved in plant growth and development as well as response to abiotic stresses. In this review, we focus on H_2O_2 signaling activities and its cross-talk with Ca^{2+} and NO in plants.

H_2O_2 HOMEOSTASIS

H_2O_2 Generation

H_2O_2 is a byproduct of aerobic metabolism in plants (Mittler, 2002). **Figure 1** shows that H_2O_2 in plants can be synthesized either enzymatically or non-enzymatically. There are numerous routes of H_2O_2 production in plant cells, such as photorespiration, electron transport chains (ETC), and redox reaction.

There is evidence for H_2O_2 production in plants through several enzymes includingcell wall peroxidases (Francoz et al., 2015), oxalate (Hu et al., 2003), amine oxidases and flavin-containing enzymes (Cona et al., 2006; **Figure 1**). Moreover, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases may also increase H_2O_2 level through generating superoxide which could be converted to H_2O_2 by superoxide dismutases (SOD; Grivennikova and Vinogradov, 2013; Brewer et al., 2015). Remans et al. (2010) observed that ROS accumulation, especially H_2O_2 formation, is mostly related with the stimulation of NADPH oxidase in plants under heavy metal stresses. Moreover, H_2O_2 produced by NADPH oxidases may significantly increase proline accumulation in *Arabidopsis thaliana* under salt or mannitol stress (Ben Rejeb et al., 2015). Additionally, some other oxidases such as glucose oxidases, glycolate oxidases (Chang and Tang, 2014), and sulfite oxidases (Brychkova et al., 2012) may oxidize their own substrates to produce H_2O_2 (**Figure 1**).

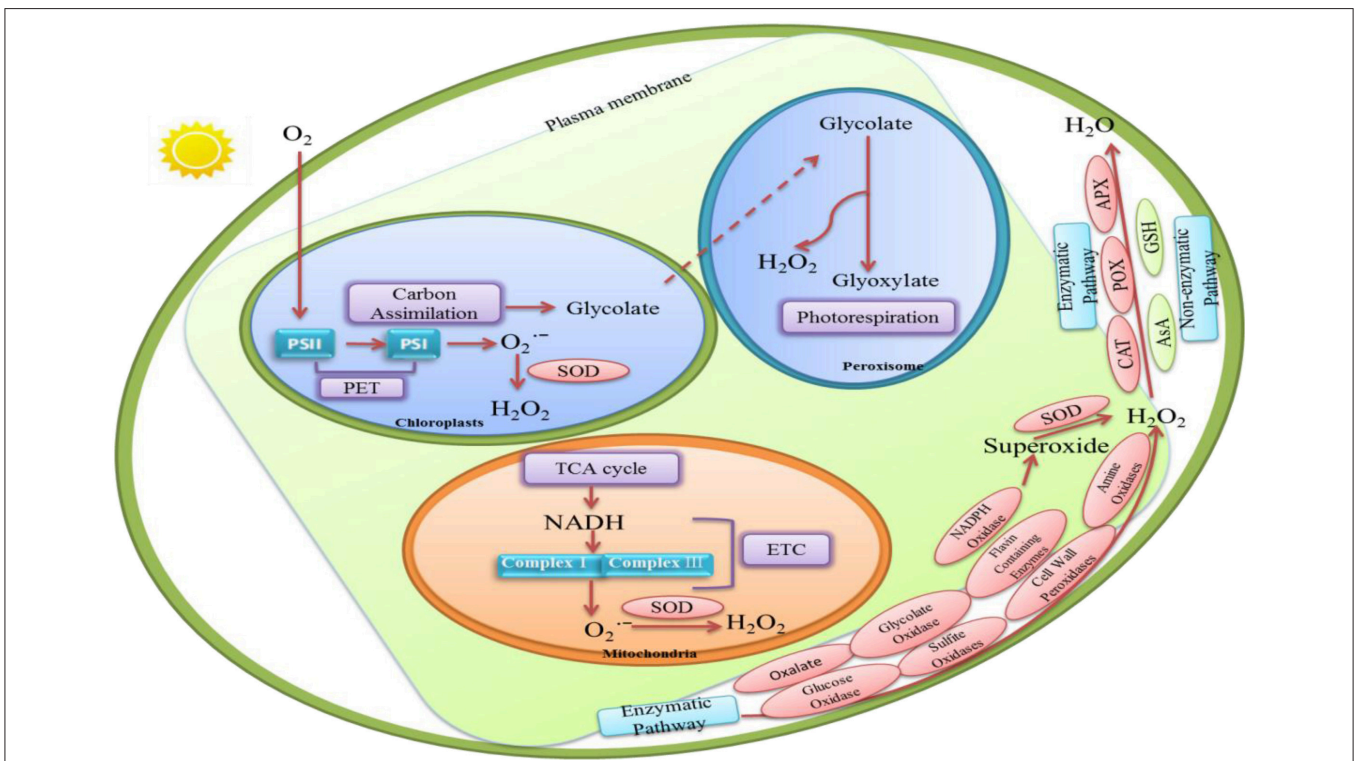

FIGURE 1 | The various routes of hydrogen perioxide (H_2O_2) production and H_2O_2 removal in plant cells. Enzymatic production of H_2O_2 in plants requires several enzymes including cell wall peroxidases (Francoz et al., 2015), oxalate (Hu et al., 2003), amine oxidases and flavin-containing enzymes (Cona et al., 2006), glucose oxidases, glycolate oxidases (Chang and Tang, 2014), and sulfite oxidases (Brychkova et al., 2012). In these enzymes, some of them may convert O_2^- to H_2O_2 and O_2. And others may oxidize their each substrates to generate H_2O_2in biocatalysis processes. Several non-enzymatic reactions are also known to produce H_2O_2. In peroxisome, H_2O_2 synthesis is associated with glycolate oxidation during photosynthetic carbon oxidation cycle (Foyer and Noctor, 2003). In chloroplasts, H_2O_2 production can be produced by the reduction of O_2^- by photosynthetic electron transport (PET) chain. H_2O_2 in chloroplast also may be detected at the manganese-containing, oxygen evolving complex which is the donor site of photosystem II. Moreover, H_2O_2 could be generated in mitochondria through aerobic respiration because O_2^- is produced from complexes I and III in the electron transport chain. H_2O_2-scavenging enzymes include catalase (CAT; Willekens et al., 1997), peroxidase (POX; Fan and Huang, 2012), ascorbate peroxidase (APX) and glutathione reductase (GR; Jahan and Anis, 2014). In non-enzymatic pathway, Ascorbate (AsA) and glutathione (GSH) are responsible for decreasing H_2O_2 level (Kapoor et al., 2015).

Several non-enzymatic reactions are also known to produce H_2O_2. For example, many reactions involved in photosynthesis and respiration are responsible for H_2O_2 production. It is generated continually via electron transport reactions both in mitochondria and chloroplasts (**Figure 1**).

Peroxisomes

Peroxisome is considered to be the site of photorespiration in plant cell, which needs light-dependent uptake of O_2 and releases CO_2 accompanying with the generation of H_2O_2. It is suggested that H_2O_2 synthesis is associated with the oxidation of glycolate during the photosynthetic carbon oxidation cycle (Foyer and Noctor, 2003; **Figure 1**).

Chloroplasts

Chloroplast is the source of photosynthesis in plants. Chloroplasts are the crucial sites for H_2O_2 production during photosynthesis. H_2O_2 generation is associated with oxygen reduction in chloroplast (**Figure 1**). Mehler (1951) discovered that reduction of O_2 lead to the formation of H_2O_2 in the presence of light in chloroplast. Moreover, H_2O_2 production can also be produced by the reduction of O_2^- by photosynthetic electron transport (PET) chain components such as Fe–S centers, reduced thioredoxin (TRX), ferredoxin and reduced plastoquinone in the chloroplast (Dat et al., 2000). In addition, non-enzymatic production of H_2O_2 in chloroplast may be detected at the manganese-containing, oxygen evolving complex which is the donor site of photosystem II (**Figure 1**). But this process, in most cases, may probably be ignored under physiological conditions.

Mitochondria

One important source of endogenously produced H_2O_2 in plant cell is mitochondria (Dickinson and Chang, 2011). H_2O_2 is generated in mitochondria during aerobic respiration when O_2^- is produced from complexes I and III in the electron transport chain, which is then rapidly converted to H_2O_2 by the enzyme superoxide dismutase (**Figure 1**).

H_2O_2 Removal

The antioxidant systems that regulate H_2O_2 levels consist of both non-enzymatic and enzymatic H_2O_2 scavengers (**Figure 1**). H_2O_2-scavenging enzymes include catalase (CAT; Willekens et al., 1997), peroxidase (POX; Fan and Huang, 2012), ascorbate peroxidase (APX) and glutathione reductase (GR; Jahan and Anis, 2014). Some studies revealed that APX was found in the cytosol (Begara-Morales et al., 2013), chloroplasts (Asada, 2006), and mitochondria (Navrot et al., 2007). Meanwhile, CAT can decompose H_2O_2 in peroxisome (Nyathi and Baker, 2006). It is quite clear that these enzymes exist in different organelles and they might decrease H_2O_2 content efficiently and maintain the stability of membranes.

Ascorbate (AsA) and glutathione (GSH), as non-enzymatic compounds, are constantly participated in regulating ROS level (Kapoor et al., 2015). AsA, a key antioxidant for elimination of H_2O_2, can react with H_2O_2 directly. GSH is a crucial antioxidant which may be associated with regenerating AsA, and rapidly oxidizes excess H_2O_2. Therefore, GSH is also involved in regulating H_2O_2 level and redox balance in plant cells (Krifka et al., 2012). In fact, H_2O_2 homeostasis seems to result in some biological effects on plant cells which may be as a signaling sign in signaling transduction pathway.

Responses to H_2O_2

Growth and Development

Table 1 shows that H_2O_2 mediates various developmental and physiological processes in plants. These findings indicate that H_2O_2 may affect different parts of plants by increasing endogenous H_2O_2 level or by regulating relative gene expression. Also, the change of H_2O_2 level may impact metabolic and antioxidant enzyme activity in favor of plant growth and development (Barba-Espín et al., 2011; Liu et al., 2013). However, the mechanisms that allow different H_2O_2 function in plants still require examination.

Stress Condition

Recent studies have demonstrated that H_2O_2 is a key signaling molecule in the signaling pathway, which associated with abiotic stress response. A number of discussions showed that H_2O_2 could respond to abiotic stresses such as drought (Hameed and Iqbal, 2014; Ashraf et al., 2015), salinity (Sathiyaraj et al., 2014; Mohamed et al., 2015), cold (Orabi et al., 2015), high temperatures (Wang Y. et al., 2014; Wu et al., 2015), UV radiation (He et al., 2005), ozone (Oksanen et al., 2004), and heavy metal (Wen et al., 2013; **Table 2**). It is clear from these studies that H_2O_2 could enhance abiotic stress resistance through protecting organelle structure under abiotic stress conditions. For instance, H_2O_2 may protect chloroplast ultrastructure to preserve photosynthesis under abiotic stress. Similarly, to improve plant abiotic stress tolerance, H_2O_2 may modulate the expression of resistance genes and antioxidant enzyme activities during abiotic stress response.

H_2O_2 as a Signaling Molecule in Plant

Among ROS, H_2O_2 has comparatively long life span and small size, which permit it to traverse through cellular membranes to different cellular compartments. García-Mata and Lamattina (2013) found that H_2O_2 may move between cells through aquaporin channels for signaling transduction. Increasing evidences point out that H_2O_2 signaling may regulate various plant physiological processes. For example, H_2O_2 as signaling molecule may participate in nitrosative stress-triggered cell death in kimchi cabbage (*Brassica rapa* var. *glabra* Regel) seedlings (Kim et al., 2015). Also, Li et al. (2015) suggested that H_2O_2 is involved in signaling crosstalk between NO and hydrogen sulfide (H_2S) to induce thermotolerance in maize seedlings. Moreover, the interaction among H_2O_2, NO and Ca^{2+} could relieve copper stress in *Ulva compressa* (González et al., 2012). H_2O_2 signaling was also demonstrated to play a salient role in brassinosteroid-regulated stomatal movement (Shi C. et al., 2015). As stated above, H_2O_2 as an important signaling molecule may play a significant role at every stage of plant life and under various abiotic stress conditions. H_2O_2 signaling appears to crosstalk with many different signaling molecules such as

TABLE 1 | The developmental and physiological effects of H_2O_2 in plants.

Developmental and physiological effect	Species	Tissue	H_2O_2 production	H_2O_2-mediated effect	References
Seed germination	*Pisum sativum* L. cv. Alaska	Seed	+	Caused carbonylation of proteins and metabolic enzyme Up-regulated *PsMAPK2* *PsMAPK3* expression	Barba-Espín et al., 2011
PCD	*Triticum aestivum* L.	Seedling	+	Increased antioxidant enzyme activities and gene expression	Cheng et al., 2015 Vavilala et al., 2015
	Chlamydomo-nas reinhardtii		+	Induced cell death Increased intracellular H_2O_2 content Increased antioxidant enzyme activities and analyses of transcripts	
Senescence	*Lilium*	Leaf	+	Increased vase life and flower diameter Reduced the degradation of RWC, total chlorophyll content and water-soluble carbohydrate	Liao et al., 2012b
Flowering	*Monilinia fructicola*	Petal	+	Increased H_2O_2 concentration Enhanced protein carbonylation (carbonyl content)and lipid peroxidation (MDA content)	Liu et al., 2013
Root system development	*Tagetes erecta* L.	Root	+	Increased root length Increased root number explant^{-1}	Liao et al., 2009
	Arabidopsis thaliana			Accelerated lateral root formation Increased endogenous H_2O_2 production Up-regulated relative expression levels of *HY1*	Ma et al., 2014
				Increased sensitivity of the root elongation zone	Hernández-Barrera et al., 2015
Stomatal closure	*Arabidopsis thaliana*	Leaf	+	Induced stomatal closure	Ge et al., 2015

hormones (Shi C. et al., 2015), protein kinase (González et al., 2012) and many other small signaling molecules (Li et al., 2015). H_2O_2 and these signaling molecules may influence each other through various positive and negative feedback loops. Thus, they co-regulate cell division and differentiation, antioxidant system as well as gene expression involved in plant development and defense.

CROSSTALK BETWEEN H_2O_2 AND NO

NO is a diatomic free radical gas. Previous studies suggested that NO could take part in a wide range of physiological processes such as vasorelaxation, nervous system, defense against pathogens in animals (Mayer and Hemmens, 1998). In mammals, NO is synthesized via three different isoforms of NO synthase (NOS) including inducible NOS (iNOS; Nathan and Hibbs, 1991), endothelial NOS (eNOS) and neuronal NOS (nNOS; Förstermann et al., 1994). In plants, NO could be synthesized through enzymatic and non-enzymatic pathways (**Figure 2**). The enzymatic pathway includes nitrate reductase (NR; Rockel et al., 2002), nitric oxide-like (NOS-like) synthase (Guo et al., 2003), Nitrite-NO reductase (Ni-NOR; Stöhr et al., 2001) and xanthine oxidase (XOR; Corpas et al., 2004) pathways. The non-enzymatic generation of NO includes nitrification or de-nitrification processes (Skiba et al., 1993, **Figure 2**).

A plethora of evidences suggest that NO, as a versatile signaling molecule, is involved in regulating every aspect of plant growth and developmental processes such as seed germination (Fan et al., 2013; Wang et al., 2015), flowering (Liu W. W. et al., 2015), root growth and development (Liao et al., 2011; Wu et al., 2014; Xiang et al., 2015), ripening and senescence (Liao et al., 2013; Shi Y. et al., 2015). Meanwhile, as a physiological regulator, NO signaling is involved in mediating stomatal closure (Noelia et al., 2015; Shi K. et al., 2015; Chen et al., 2016), pollen tube growth (Wang et al., 2009). Also, NO plays an essential role in plant disease resistance (Rasul et al., 2012; Kovacs et al., 2015) and responses to various abiotic stresses such as cold (Fan et al., 2015), heat (Yu et al., 2015), salt (Liu W. et al., 2015), drought (Shan et al., 2015), UV-B (Esringu et al., 2015) and heavy metal (Alemayehu et al., 2015; Chen et al., 2015; Kaur et al., 2015).These studies have paved the way to understand the signaling roles of NO which may affect cell metabolism, cellular redox balance and gene expression in plants. The relative target receptor may receive signaling activated by various stimuli. As a result, NO may activate regulatory mechanism to promote developmental and physiological processes and regulate abiotic stress response in plants.

TABLE 2 | Report on H_2O_2-mediated effect during stresses in plants.

Stress	Plant species	Tissue	H_2O_2-mediated effect	References
Drought	*Triticum aestivum* L.	Leaf	Increased SOD, POD, CAT activities Raised total phenolic and reducing sugars content	Hameed and Iqbal, 2014
	Zea mays L.	Leaf	Reduced degradation of chlorophyll increased endogenous H_2O_2, MDA contents Increased antioxidant enzymes activities Increased ascorbic acid content and ion contents	Ashraf et al., 2015
Salt	*Panax ginseng*	Leaf	Increased chlorophyll and carotenoid content Increased Relative water content Increased growth height and dry-weight Increased antioxidant activity Up-regulated relative gene expression of defense related genes	Sathiyaraj et al., 2014
	Lycopersicon esculentum L.		Decreased electrolyte leakage Increased endogenous H_2O_2 and MDA content Increased antioxidant enzymes activities Affect protein pattern and peroxidase enzymes	Mohamed et al., 2015
Cold	*Lycopersicon esculentum* L.	Seedling	Increased antioxidant enzymes activities Increased MDA content Decreased electrolyte leakage Increased total soluble solids	Orabi et al., 2015
Heat	*Festuca arundinaceaLolium perenne*	Leaf	Decreased the GSH/GSSG ratio Increased POD, CAT, APC, GR, and GPX activities	Wang Y. et al., 2014
	Arabidopsis thaliana	Seedling	Increased thermotolerance Enhanced antioxidant enzyme activities Increased endogenous NO content Increased HSFs activity and HSP21 accumulation	Wu et al., 2015
UV-B	*Vicia faba* L.	Leaf	Increased endogenous H_2O_2 production Induced Stomatal closure	He et al., 2005
Ozone	*Betula papyrifera*	Leaf	Induced proliferation of peroxisomes Increased Level of gene expression for catalase (*Cat*)	Oksanen et al., 2004
Heavy metal	*Zea mays var.* rugosa Bonaf	Seedling	Decreased the activities of proline dehydrogenase Increased the activities of Arginase and OAT, P5CS and GDH Up-regulated the expression levels of *P5CS*, *GDH*, *Arginase, OAT* and *ProDH* genes	Wen et al., 2013

Interaction in Growth and Development

To date, the interaction between H_2O_2 and NO has been demonstrated clearly in plants. The signaling crosstalk between H_2O_2 and NO has been considered to be an essential factor to influence plant developmental and physiological processes such as leaf cell death (Lin et al., 2012), delay senescence (Iakimova and Woltering, 2015), root growth and development (Liao et al., 2010, 2011), stomatal closure (Huang et al., 2015; Shi K. et al., 2015), and pollen tube growth (Serrano et al., 2012). **Table 3** shows the interaction of H_2O_2 and NO at different levels in a great number of developmental and physiological processes in plants. On the one side, H_2O_2 may act as a cofactor to promote endogenous NO synthesis. For example, Lin et al. (2012) implied that H_2O_2 may stimulate NO production through increasing NR activity in leaves of *noe1* plants under high light. Shi C. et al. (2015) reported that Gα-activated H_2O_2 production may induce NO synthesis. The research found that NO could modulate stomatal closure in H_2O_2 mutants *AtrbohF* and *AtrbohD AtrbohF* and in the wild type treated with H_2O_2 scavenger and inhibitor. However, H_2O_2 did not close or reduce the stomatal closure in mutants *Nia1-2* and *Nia2-5 Nia1-2*, and in the wild type treated c-PTIO or tungstate (Shi C. et al., 2015). These results clearly show that H_2O_2 might induce NO synthesis in stomatal closure. On the other side, NO may induce H_2O_2 generation in plants. Liao et al. (2011) reported cPTIO or L-NAME could inhibit the endogenous H_2O_2 generation implying that NO was required for the production of H_2O_2 during adventitious rooting. Meanwhile, NO could mediate antioxidant enzyme activities to influence the H_2O_2 level (Zhang et al., 2007). Thus, the interaction of H_2O_2 and NO may trigger a serious of physiological and biological response in plant cells.

Interaction during Abiotic Stress

Recently, the roles of $H_2O_2$and NO signaling and their crosstalk in mediating plant response to abiotic stresses have been largely established (**Table 4**).

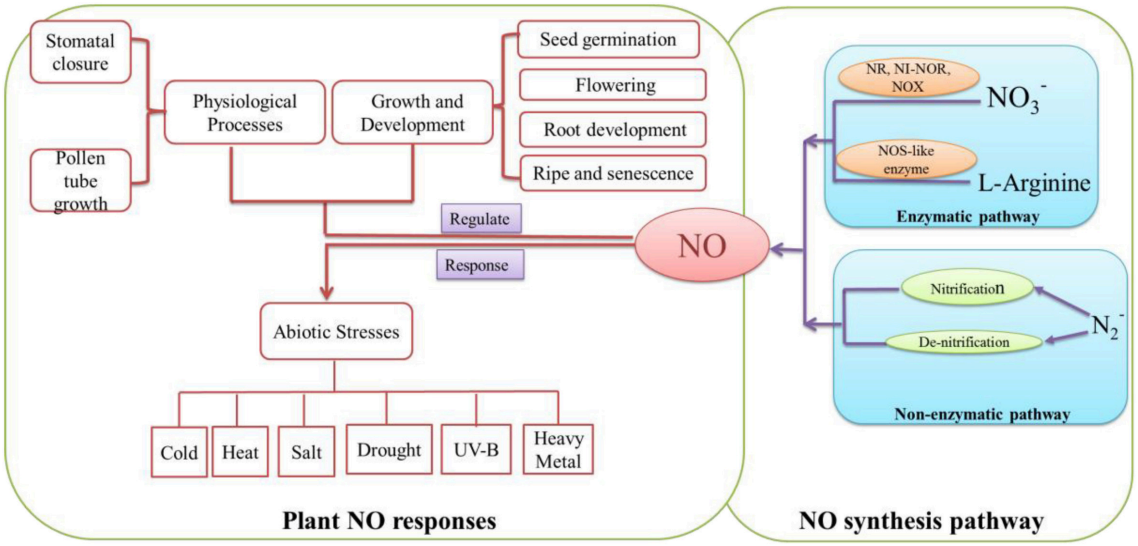

FIGURE 2 | Summary of the main NO systhetic pathways and NO functions in plant growth, development and defense processes. NO may be synthesized by enzymatically and non-enzymatically pathways. In enzymatic pathway, nitrate reductase (NR; Rockel et al., 2002), Nitrite-NO reductase (Ni-NOR; Stöhr et al., 2001) and xanthine oxidase (XOR; Corpas et al., 2004) could convert NO_3^- and NO_2^- to NO. Meanwhile, because of NOS-like enzyme (Guo et al., 2003), L-Arginine may be catalyzed to NO. In non-enzymatic pathway, N_2^- could be transformed to NO through nitrification and denitrification (Skiba et al., 1993). NO plays an important signaling molecule in plant. It could regulate developmental and physiological processes such as seed germination (Wang et al., 2015), root development (Liao et al., 2011) and stomatal closure (Shi C. et al., 2015). Also, it may be involved in response to abiotic stresses such as cold (Fan et al., 2015), salt (Liu W. et al., 2015) and drought (Shan et al., 2015).

TABLE 3 | The developmental and physiological effects of crosstalk between H_2O_2 and NO in plants.

Developmental and physiological effect	Species	Tissue	Crosstalk between H_2O_2 and NO mediated effects	References
Cell death	*Oryza sativa*	Leaf	H_2O_2 induced NR-dependent NO generation NO Is required for H_2O_2-induced leaf cell death increased NR enzyme	Lin et al., 2012
Senescence	*Lactuca sativa* L.	Leaf	NO decreased endogenous H_2O_2 content Delay senescence	Iakimova and Woltering, 2015
Root growth	*Dendranthema morifolium*	Root	Increase the activities of PPO, IAAO and the content of WSC and total nitrogen Decrease the total polyphenol content NO and H_2O_2 may act synergistically to mediate adventitious root generation and development	Liao et al., 2010 Liao et al., 2011
	Tagetes erecta L.		NO may be involved as an upstream signaling molecule for H_2O_2 production	
Pollen tube growth	*Olea europaea* L.	Flower	Decreased cell death Increased nitrated proteins	Serrano et al., 2012
Stomatal movement	*Arabidopsis*	Leaf	H_2O_2 production was required for NO synthesis Regulated stomatal closure	Shi C. et al., 2015 Huang et al., 2015
	Vicia faba		Regulated stomatal closure H_2O_2 induced NO production	

Drought

Drought stress is a major environmental factor that affects plant growth and development. As reported by Liao et al. (2012a), both H_2O_2 and NO could protect mesophyll cells ultrastructure and improve the photosynthetic level of leaves under drought stress during adventitious rooting in marigold explants. Similarly, the interplay between H_2O_2 and NO signaling may increase the activity of myo-inositol phosphate synthase to alleviate drought stress (Tan et al., 2013). Additionally, Lu et al. (2009) suggested that endogenous NO and H_2O_2 may be involved in ABA-induced drought tolerance of bermudagrass by increasing antioxidant enzyme activities. NO may be considered to be upstream or downstream signaling molecule of H_2O_2 (Lu et al., 2009; Liao et al., 2012a). Thus, the interaction between H_2O_2 and NO

TABLE 4 | Reports on interaction between H_2O_2 and NO involved in abiotic stresses in plants.

Stress	Plant species	Tissue	Crosstalk between H_2O_2 and NO mediated effects	Reference
Salt	*Citrus aurantium* L.	Leaf	Alleviated salinity-induced protein carbonylation Shifted the accumulation levels of leaf S-nitrosylated proteins acclimation to salinity Identified a number of proteins which were modulated by both H_2O_2 and NO treatments	Tanou et al., 2009 Tanou et al., 2010
	Populus euphratica	shoot	Increased K/Na ratio	Zhang et al., 2007
	Medicago falcata	Seedling	Stimulated expression of PM H^+-ATPase Induced *MfMIPSI* transcript Increased the level of myo-inositol	Tan et al., 2013
Drought	*Tagetes erecta* L.	Root	Alleviated the destruction of mesophyll cell ultrastructure Increased leaf chlorophyll content Mediated chlorophyll fluorescence parameters Enhanced carbohydrate accumulation Decreased starch content H_2O_2 generation may be affected by NO	Liao et al., 2012a
	Tagetes erecta L.	Leaf	Increased RWC Decrease ion leakage Increased antioxidant enzyme, PEPCase, HK activities and MDA content NO acted downstream of H_2O_2	Lu et al., 2009
UV-B	*Arabidopsis*	Leaf	NO production depends on H_2O_2 Mediated stomatal closure	He et al., 2013
			The UV-B Photoreceptor UVR8 was mediated by H_2O_2 and NO	Tossi et al., 2014
Heat	*Zea may* L.	seedling	Improved survival percentage of maize seedlings H_2O_2 increased endogenous NO content H_2O_2 may be involved in downstream signal of NO	Li et al., 2015
	Arabidopsis		NO is involved in H_2O_2 signaling as a downstream factor. Increased HS factor activity and HS protein accumulation.	Wang L. et al., 2014
	Triticum aestivum L.		Increased seedling resistance Increased H_2O_2 and NO content Increased survival percentage of seedlings	Karpets et al., 2015
Cold	*Medicago sativa* subsp. falcata	Leaf	Mediated cold-induced *MfSAMS1* expression	Guo et al., 2014
	Medicago falcate *Medicago sativa*	Seedling	Up-regulated *MfMIPSI* expression Increased myo-inositol content	Tan et al., 2013
Heavy metal	*Ulva compressa*	Cell	Increased PDH,IDH,OGDH activity and increased relative transcript levels	González et al., 2012
	Triticum aestivum	Root	Decreased lipid peroxidation Increased NOS activity Increased antioxidative enzyme activities	Duan et al., 2015

may alleviate drought stress through up-regulating antioxidant defense system to protect cell membrane and maintain ion homeostasis in plants.

Salt

The interaction between H_2O_2 and NO plays an important role in plant tolerance to salt stress (Zhang et al., 2007; Tan et al., 2013). Tanou et al. (2009) suggested that H_2O_2 and NO pre-treatments could alleviate salinity-induced protein carbonylation in citrus. The authors suggested an interaction between H_2O_2 and NO during salt stress response. Furthermore, H_2O_2- and NO-responsive proteins have been identified which may further reveal a protein interaction network between H_2O_2 and NO signaling under salt stress (Tanou et al., 2010).

UV-B

UV-B, a key environmental signal, initiates diverse responses in plants (Jansen and Bornman, 2012).UV-B radiation can also influence plant growth, development, and productivity. It has been shown that the crosstalk between H_2O_2 and NO could be involved in the response to UV-B stress. There was an interrelationship among Gα protein, H_2O_2, and NO during UV-B-induced stomatal closure in *Arabidopsis* leaves (He et al., 2013). This study found that there was a significant increase in H_2O_2 or NO levels which associated with stomatal closure in the wild type by UV-B stress. However, these effects were abolished by double mutants of *AtrbohD* and *AtrbohF* or *Nia*1 mutants. These results strongly suggested that the crosstalk between H_2O_2 and NO signaling might play an essential role during UV-B-induced stomatal closure in guard cells. Recently, Tossi et al. (2014) also

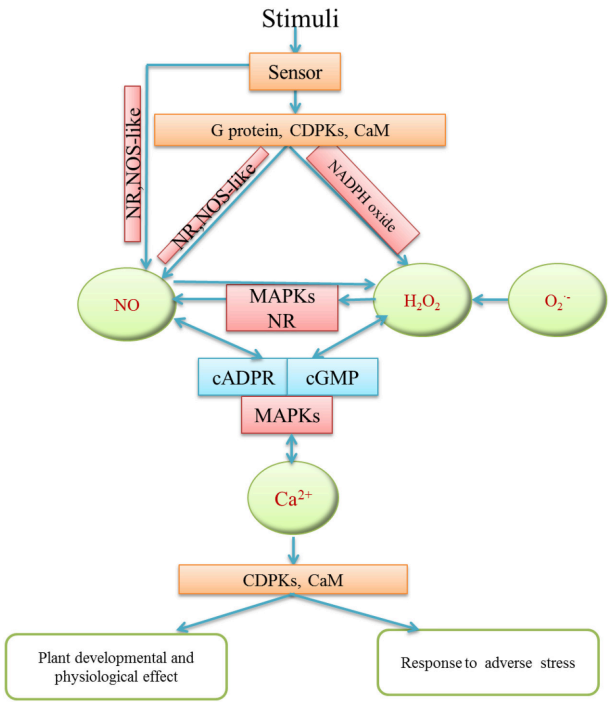

FIGURE 3 | Schematic model of the interaction among H_2O_2, NO, Ca^{2+} in different plant physiological and defense processes. H_2O_2, NO and Ca^{2+} may receive various stimuli through signaling sensors. They might interact via cross-regulation and transduce signaling to downstream molecules by activating phosphokinase like MAPKs, or relative enzyme activity in order to regulate plant development and growth and abiotic stress responses.

showed a mechanism involving both H_2O_2 and NO generation in response to UV-B exposure. Therefore, the crosstalk between H_2O_2 and NO can regulate stomatal movement to reduce UV-B stress damage to plant cells.

Cold

Cold stress adversely influences plant growth and development. Guo et al. (2014) reported that the interaction of H_2O_2 and NO may affect cold-induced S-adenosylmethionine synthetase and increase cold tolerance through up-regulating polyamine oxidation in *Medicago sativa* subsp. *falcate*. Moreover, signaling interplay of H_2O_2 and NO was essential for cold-induced gene expression of falcata myo-inositol phosphate synthase (*MfMIPS*), which improved tolerance to cold stress (Tan et al., 2013). Thus, the interaction between H_2O_2 and NO may initiate different mechanisms to response to cold stresses.

Heat

Recently, many studies have been conducted to investigate the relationship between H_2O_2 and NO under heat stress. Li et al. (2015) reported that a signaling crosstalk between H_2O_2 and NO may be involved in inducing thermotolerance in maize seedlings. Moreover, H_2O_2 may be upstream signaling of NO in the heat shock pathway in *Arabidopsis* seedlings (Wang L. et al., 2014). In addition, treatment with low level of H_2O_2 or NO could increase seedling viability under heat resistance (Karpets et al., 2015). These studies support the existence of crosstalk between H_2O_2 and NO in heat responses in plants.

Heavy Metal Stress

Alberto et al. (2012) suggested that the signaling interaction between H_2O_2 and NO was involved in alleviating copper stress of *Ulva compressa* through mediating antioxidant enzyme activities and activating relative gene expression. Besides, the interplay of NO and H_2O_2 in wheat seedlings participated in regulating root growth under zinc stress and alleviated zinc stress through increasing antioxidant system, decreasing lipid peroxidation as well as up-regulating resistance gene expression (Duan et al., 2015). Obviously, the crosstalk of H_2O_2 and NO has been found under heavy metal stress condition, which may trigger a variety of antioxidant responses in plants.

As stated above, the physiological effect of H_2O_2 and NO is similar and synergetic. In different cases, these forms of interaction are various. However, the form of H_2O_2 and NO crosstalk depend on plant species and environmental stresses. H_2O_2 and NO could modulate each other through regulating antioxidant enzymes activities and relative gene expression in plants. Meanwhile, H_2O_2 and NO may synergistically regulate many common target genes which were related to signaling transduction, defense reaction, plant hormone interactions, protein transport and metabolism. Therefore, it has a significant meaning to elaborate the mechanism of the interaction between H_2O_2 and NO in plant developmental processes and response to abiotic stresses.

CROSSTALK BETWEEN H_2O_2 AND CA^{2+}

Ca^{2+} is a widespread signaling molecule in plants. When plants receive stimuli, the change of intracellular Ca^{2+} concentration may transfer signaling to regulate a series of cellular processes in plants (Kong et al., 2015; Tang et al., 2015). There are various types of Ca^{2+} receptors and channels in plants such as Ca^{2+}-ATPases (Pászty et al., 2015), Ca^{2+}-binding sensor protein (Wagner et al., 2015), inositol-1,4,5-trisphosphate (IP_3; Serrano et al., 2015) and cyclic ADP-ribose (cADPR, Gerasimenko et al., 2015). It is well known that Ca^{2+} is involved in plant growth and development such as seed germination (Kong et al., 2015), pollen tube growth (Zhou et al., 2014), leaf de-etiolation (Huang et al., 2012), root growth and development (Liao et al., 2012a; Han et al., 2015) and other physiological processes including cell polarity regulation (Zhou et al., 2014; Himschoot et al., 2015), stomatal closure (Zou et al., 2015) and immune response (Seybold et al., 2014). Furthermore, variations in cytosolic free Ca^{2+} concentration have been demonstrated to response to a wide range of environmental stresses such as heat shock (Urao et al., 1994), drought (Zou et al., 2015), light (Hu et al., 2015), salt (Tepe and Aydemir, 2015), and heavy metal (Li et al., 2016). Because of Ca^{2+} has various receptors and channels in plants, it may receive different upstream signaling molecules quickly and then respond to abiotic stress.

Interaction in Growth and Development

Crosstalk between H_2O_2 and Ca^{2+} occurs in plant cells (**Table 5**). For example, exogenous H_2O_2 caused transiently dose-dependent increase in Ca^{2+} influx in *Arabidopsis thaliana* root epidermis (Demidchik et al., 2007). Two Ca^{2+} channels could be regulated by H_2O_2 level in root elongation zone. Han et al. (2015) demonstrated that H_2O_2 signaling could induce root elongation by mediating Ca^{2+} influx in the plasma membrane of root cells in *Arabidopsis* seedlings. Richards et al. (2014) also suggested that Annexin 1, a Ca^{2+} transport protein, may regulate H_2O_2-induced Ca^{2+} signature in *Arabidopsis thaliana* roots to promote root growth and development. Additionally, Ca^{2+} signaling was involved in H_2O_2-induced adventitious rooting in marigold because removal of Ca^{2+} could inhibit H_2O_2-induced adventitious root development (Liao et al., 2012a). Interestingly, Wu et al. (2010)'s findings strongly suggested that spermidine oxidase (Spd)-derived H_2O_2 signaling may mediate Ca^{2+} influx. Spd was probably related to downstream induction of H_2O_2 signaling and then H_2O_2 activated Ca^{2+}-permeable channels during pollen tube growth (Wu et al., 2010). Cross talk between Ca^{2+}–Calmodulin (CaM) and H_2O_2 also played a significant role in antioxidant defense in ABA signaling in maize leaves (Hu et al., 2007; **Table 5**). Thus, the signaling crosstalk between H_2O_2 and Ca^{2+} may affect every stage of plant development by modulating cell elongation and division, antioxidant enzyme activity and gene expression. H_2O_2 may activate Ca^{2+} receptors and target proteins to increase $[Ca^{2+}]_{cyt}$ level and Ca^{2+} may induce endogenous H_2O_2 generation during plant growth and development.

Interaction in Abiotic Stress

Clearly, correlations also exist between H_2O_2 and Ca^{2+} in response to abiotic stresses in plants (**Table 6**). Shoresh et al. (2011) investigated that supplemental Ca^{2+} had a significant effect on H_2O_2 metabolism and regulating leaves and roots growth in maize under salt stress. The authors indicated that extracellular Ca^{2+} may modulate endogenous H_2O_2 levels through activating polyamine oxidase activity. Also, salt stress may induce H_2O_2 accumulation in Ca^{2+}-dependent salt resistance pathway in *Arabidopsis thaliana* roots (Li et al., 2011). Moreover, Lu et al. (2013) suggested that exogenous H_2O_2 and Ca^{2+} may mediate root ion fluxes in mangrove species under NaCl stress. Obviously, H_2O_2 may interact with Ca^{2+} under salt stress in plants through mediating root ion balance, increasing antioxidant enzymatic activity and up-regulating the expression of related genes. Moreover, H_2O_2 and Ca^{2+} signaling were also involved in ABA responses to drought stress in *Arabidopsis thaliana* through Ca^{2+}-dependent protein kinase8 (CPK8) which could regulate catalase3 (CAT3) activity mediating stomatal movement (Zou et al., 2015). In addition, Qiao et al. (2015) reported that a Ca^{2+}-binding protein (rice annexin OsANN1) could enhance heat stress tolerance by modulating H_2O_2 production. Over production of H_2O_2 induced by heat stress increased *OsANN*1 expression and up-regulated the level of *SOD* and *CAT* expression, which constructed a signaling mechanism for stress defense in plants (Qiao et al., 2015). Until now, the signaling crosstalk between H_2O_2 and Ca^{2+} may regulate various responses to abiotic stresses in plants. It may be connected with the regulation of antioxidant system. Thus, the interaction between H_2O_2 and Ca^{2+} may increase antioxidant enzyme activities such as APX, SOD, and GR. These antioxidant enzymes may alleviate stress damages in plants. In addition, the crosstalk between H_2O_2 and Ca^{2+} could regulate gene expression level and induce protein interactions.

It appears that the interrelationship between H_2O_2 and Ca^{2+} may be involved in various aspects of plant growth and development processes and abiotic stress responses. In fact, the change of Ca^{2+} concentration is closely related to H_2O_2 burst in plant cells. The combination of H_2O_2 and Ca^{2+} may play crucial roles in plants. Different plants even different parts of the same plant may have different modulation mechanisms. Thus, relationship between H_2O_2 and Ca^{2+} signaling in plants is very complex. The interplay of H_2O_2, Ca^{2+} and its mechanism need to be illustrated clearly in the future.

CROSSTALK AMONG H_2O_2, NO AND CA^{2+}

It has been suggested that there is a connection among H_2O_2, NO,and Ca^{2+} in plants. H_2O_2, NO, and Ca^{2+} may act as essential signaling molecules which may form a complex signaling network to regulate different developmental and physiological processes in plants (**Figure 3**). For instance, during adventitious rooting of mung bean, Ca^{2+} signaling played a pivotal role and functioned as a downstream molecule of H_2O_2 and NO signal pathway (Li and Xue, 2010; **Figure 3**). Similarly, there is a possible relationship among H_2O_2, NO and Ca^{2+}/CaM during adventitious rooting in marigold explants (Liao et al., 2012a). The authors found that exogenous NO and H_2O_2 promoted adventitious root development in marigold explants through increasing endogenous Ca^{2+} and CaM levels. Moreover, H_2O_2, NO and Ca^{2+} were also involved in oligochitosan-induced programmed cell death in tobacco suspension cells (Zhang et al., 2012). Pharmacological experiments revealed that Ca^{2+} signaling induced NO accumulation through inducing H_2O_2 generation during stomatal closure in *Arabidopsis* guard cells (Li et al., 2009). Furthermore, Wang et al. (2011) suggested a functional correlationship among H_2O_2, calcium-sensing receptor (CAS) and NO in Ca^{2+}-dependent guard cell signaling. It was shown that CAS may transduce Ca^{2+} signaling through activating its downstream target NO and H_2O_2 signaling pathway (Wang et al., 2011). Therefore, it is thus clear that the interplay of H_2O_2, NO, and Ca^{2+} may have an significant effect on plant growth and physiological processes through promoting cell proliferation, controlling cell metabolism, meanwhile, regulating modes of cell death. Moreover, Vandelle et al. (2006) has reported that NO and H_2O_2 synthesis could also act upstream to increase cytosolic Ca^{2+} concentration during hypersensitive response (HR) through activating plasma membrane- and intracellular membrane-associated Ca^{2+} channels. Besides, the interaction among H_2O_2, NO, and Ca^{2+} signaling may regulate ABA-induced antioxidant defense in maize (Ma et al., 2012). Obviously, the mutual effect among H_2O_2, NO and Ca^{2+}

TABLE 5 | The developmental and physiological effects of crosstalk between H_2O_2 and Ca^{2+} in plants.

Developmental and physiological effect	Species	Tissue	Crosstalk between H_2O_2 and Ca^{2+} mediated effects	References
Root growth and elongation	*Arabidopsis*	Root	H_2O_2 induce Ca^{2+} influx Increased root elongation Endogenous H_2O_2 resulted in Ca^{2+} flux Enhanced root growth	Han et al., 2015 Demidchik et al., 2007
Adventitious root development	*Arabidopsis*	Root	Extracellular H_2O_2 induced a sustained increase in cytosolic free Ca^{2+} Exogenous H_2O_2 induced expression of *AtANN1*	Richards et al., 2014 Liao et al., 2012a
	Tagetes erecta L.		Endogenous H_2O_2 increased Cytosolic free Ca^{2+} and CaM content Induced adventitious root development	
Pollen growth	*P.Dyrifolia Nakai* cv.Hosui Imamuraaki	Flower	H_2O_2 activates Ca^{2+} currents Induced pollen tube growth	Wu et al., 2010
Antioxidant defense	*Zea may* L.	Leaf	H_2O_2 increased the concentration of cytosolic Ca^{2+} in the protoplasts of mesophyll cells and the expression of the calmodulin 1 (*CaM1*) gene and CaM content in leaves Enhanced the expression of the antioxidant genes	Hu et al., 2007

TABLE 6 | Reports on interaction between H_2O_2 and Ca^{2+} involved in abiotic stresses in plants.

Stress	Plant species	Tissue	Crosstalk between H_2O_2 and Ca^{2+} mediated effects	References
Salt	*Bruguiera gymnorrhiza* L. *Kandelia candel* L.	RootLeaf	Mediated root ion flux Increased K^+ flux and Na^+/H^+ antiport	Lu et al., 2013
	Arabidopsis	Root	Increased NADPH/$NADP^+$,G6PDH activity Up-regulated expression of PM H^+-ATPase gene	Li et al., 2011
Drought	*Zea may* L.	Root	Increased root viability Decreased membrane leakage Increased chlorophyll content Increased peroxidase activity	Shoresh et al., 2011
	Arabidopsis	Seedling	Induced stomatal closure Mediated protein interaction between CPK8 and CAT3	Zou et al., 2015
Heat	*Oryza sativa* subsp. japonica	Seedling	Up-regulated *OsANN1* expression Enhanced the level of *SOD*, *CAT* expression	Qiao et al., 2015

may increase antioxidant system and induce disease defense in plants.

Furthermore, the interplay among H_2O_2, NO, and Ca^{2+} also have an effect on abiotic stress response in plants. For example, Lang et al. (2014) reported that NO likely interacted with Ca^{2+} and H_2O_2 in *Aegiceras corniculatum* to up-regulate Na^+/H^+ antiport system of plasma membrane under salt stress. There were species-specific interactions between H_2O_2, Ca^{2+}, NO, and ATP in salt-induced reduction of K^+ efflux (Lang et al., 2014). Moreover, there was a crosstalk among H_2O_2, NO, and Ca^{2+} when *Ulva compressa* exposed to copper excess and the interaction had a significant effect on transcriptional activation of target genes (Alberto et al., 2012). The H_2O_2-induced NO generation could be inhibited by Ca^{2+} channel blockers, implicating that Ca^{2+} may mediate the effect of H_2O_2 on NO production. Furthermore, Ca^{2+} release through different type of Ca^{2+} channels was also shown to be activated by NO and H_2O_2 (Alberto et al., 2012; **Figure 3**). The interrelationship between H_2O_2, NO and Ca^{2+} may provide additional layers of responses to abiotic stresses through controlling ion transport, increasing antioxidant enzyme activities and affecting expression of resistance genes, indicating a feedback mechanism between H_2O_2, NO and Ca^{2+} under abiotic stresses. In a word, the combination of these findings strongly supports the view that there has an interaction among H_2O_2, NO, and Ca^{2+} signaling pathway in plant growth, development and abiotic stress responses. During signaling transduction, Ca^{2+} signaling could be activated by H_2O_2 and NO; it could also regulate H_2O_2 and NO signaling. Ca^{2+} may act as a point of signaling convergence between H_2O_2 and NO signaling pathways in plants. However, the network of H_2O_2, NO, and Ca^{2+} seems to be intricate and multidimensional. Therefore, considerably more work will need to be done to determine the interaction among H_2O_2, NO and Ca^{2+} signaling in plants.

CONCLUSION

H_2O_2 was once considered as a poisonous molecule in plants. Based on current studies, H_2O_2 may be a vital signaling molecule which controls plant growth and development. Interestingly, NO and Ca^{2+} which also act as the key component of signaling transduction in plants seem to be as upstream or downstream signaling molecules of H_2O_2. Meanwhile, H_2O_2 modulates NO and Ca^{2+} signaling pathways. There is a complex interactive network among H_2O_2, NO, and Ca^{2+} in plants. Moreover, the interplay among them has functional implications for regulating developmental and physiological processes which may increase the possibility of signal reception and transduction in plants. Future work will need to focus on the molecular mechanism of the interplay among H_2O_2, NO, and Ca^{2+} during signaling transduction in plants.

AUTHOR CONTRIBUTIONS

LN wrote the paper. WL provided the idea and revised the paper.

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Identification and Comparative Analysis of H_2O_2-Scavenging Enzymes (Ascorbate Peroxidase and Glutathione Peroxidase) in Selected Plants Employing Bioinformatics Approaches

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Among major reactive oxygen species (ROS), hydrogen peroxide (H_2O_2) exhibits dual roles in plant metabolism. Low levels of H_2O_2 modulate many biological/physiological processes in plants; whereas, its high level can cause damage to cell structures, having severe consequences. Thus, steady-state level of cellular H_2O_2 must be tightly regulated. Glutathione peroxidases (GPX) and ascorbate peroxidase (APX) are two major ROS-scavenging enzymes which catalyze the reduction of H_2O_2 in order to prevent potential H_2O_2-derived cellular damage. Employing bioinformatics approaches, this study presents a comparative evaluation of both GPX and APX in 18 different plant species, and provides valuable insights into the nature and complex regulation of these enzymes. Herein, (a) potential GPX and APX genes/proteins from 18 different plant species were identified, (b) their exon/intron organization were analyzed, (c) detailed information about their physicochemical properties were provided, (d) conserved motif signatures of GPX and APX were identified, (e) their phylogenetic trees and 3D models were constructed, (f) protein-protein interaction networks were generated, and finally (g) GPX and APX gene expression profiles were analyzed. Study outcomes enlightened GPX and APX as major H_2O_2-scavenging enzymes at their structural and functional levels, which could be used in future studies in the current direction.

Keywords: ROS, signal transduction, antioxidant, peroxisome, chloroplast, mitochondria

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INTRODUCTION

Reactive oxygen species (ROS), once perceived as toxic by-products, were known to cause oxidative damage in cells (Mittler et al., 2004; Suzuki and Mittler, 2006). Later, novel regulatory roles of these species were revealed in a wide range of biological processes such as cell signaling, growth, development, programmed cell death, and plant responses to various biotic/abiotic stress factors (Mullineaux and Karpinski, 2002; Uzilday et al., 2014). H_2O_2 is an endogenous ROS species known to play a dual role in plants, where it is beneficial at low concentrations but lethal at higher levels (Petrov and Van Breusegem, 2012). Nevertheless, at steady state levels, H_2O_2 acts as signaling molecule inducing the signal transduction mechanism to produce various cellular responses. Interestingly, pre-treatment of plants with H_2O_2 makes them more tolerant to biotic/abiotic stresses (Hossain et al., 2015). H_2O_2 was also noted for its regulatory functions in photosynthesis, cell cycle, development, senescence, and apoptosis (Mittler et al., 2004; Petrov and Van Breusegem, 2012). H_2O_2 has been accepted as a central component of signal transduction pathways in plant-adaptation to altered environmental conditions as it is both the only ROS with high permeability across membranes (that enables the transport of signals to distant sites) and its high stability when compared to other ROS with ∼1 ms half-life (Bienert et al., 2007; Dynowski et al., 2008; Petrov and Van Breusegem, 2012). On the other hand, when the delicate balance between production and scavenging of H_2O_2 is disturbed, its overproduction results in significant damage to cell structures (Anjum et al., 2015; Sofo et al., 2015). To overcome H_2O_2-related cellular damage, aerobic organisms have developed various antioxidant machineries with enzymatic and non-enzymatic components. Ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) are the main enzymes responsible for suppressing toxic levels of H_2O_2 (Apel and Hirt, 2004). However, APX may have pivotal roles in ROS-scavenging because even very low concentrations are sufficient for H_2O_2 decomposition (Anjum et al., 2014; Sofo et al., 2015).

APX (EC, 1.11.1.11) belongs to the plant-type heme peroxidase superfamily in plants (Lazzarotto et al., 2011). Genome-wide studies demonstrated that APX in higher plants is encoded by multigenic families. *Arabidopsis* was reported to contain nine APX genes; whereas, rice has eight and tomato seven (Chew et al., 2003; Teixeira et al., 2004; Najami et al., 2008). Different isoforms are classified into sub-families according to their subcellular localization. Transmembrane domains in N- and C- terminal regions, as well as organelle-specific target molecules are the primary determinants in target localization of APXs (Ishikawa et al., 1998; Negi, 2011). Among nine *APX* genes identified in *Arabidopsis*, three were found to be encoded in cytosol whereas the other six were distributed in stroma, thylakoid, and peroxisome (Chew et al., 2003; Mittler et al., 2004). In rice, chloroplastic isoforms were expressed by three genes, cytosolic and peroxisomal forms were both encoded by two genes, and one gene was for the mitochondrial APX (Teixeira et al., 2006; Anjum et al., 2014). APX activity was also reported to increase under various stress conditions. For example, APX is differentially upregulated in response to heavy metal, drought, water, and heat stress (Sharma and Dubey, 2005; Koussevitzky et al., 2008; Yang et al., 2008; Anjum et al., 2014). In a previous study, Arg-38, Glu-65, Asn-71, and Asp208 residues were reported to be conserved among the entire APX family and known to be important in ligand (heme)-binding (Welinder, 1992). In addition to enzymatic properties, structural investigations on catalytic domains of the enzymes have been also performed. Three-dimensional structures of cAPX, sAPX, and their substrates showed the relationship between loop structure and stability in the absence of ascorbate (AsA; Yabuta et al., 2000; Anjum et al., 2014). The mitochondrial and chloroplastic APXs (<30 s) have shorter half inactivation times (>1 h) compared to cytosolic and peroxisomal isoforms, which makes them more sensitive in either low concentrations or the absence of AsA (Caverzan et al., 2012; Anjum et al., 2014). Another important enzyme in H_2O_2-scavenging is the GPX from the non-heme containing peroxidase family (Bela et al., 2015). In *Arabidopsis*, eight *GPX* genes were reported (Milla et al., 2003; Koua et al., 2009). Based on *in silico* analysis, GPXs were predicted in chloroplast, mitochondria, cytosol, and ER localizations (Rouhier and Jacquot, 2005), and demonstrated high level of sequence similarity with strictly conserved cysteines and motifs (Dietz, 2011). Plant GPXs have cysteine residue in their active site (Koua et al., 2009), which is functional in both glutathione (GSH) and thiol peroxidase classes of the non-heme family. GPXs were also reported to be involved in stress responses. Many studies have demonstrated the significant increase in mRNA levels of GPXs under various abiotic/abiotic stress conditions such as oxidative stress, pathogen attack, metal, cold, drought, and salt (Navrot et al., 2006; Diao et al., 2014; Fu, 2014; Gao et al., 2014). For example, GPX genes were found to be upregulated under excess H_2O_2 and cold stresses in rice (Passaia et al., 2013). Transcriptome analysis indicated high level of GPX transcripts in dehydrated *Glycine max* samples (Criqui et al., 1992; Ferreira Neto et al., 2013). Several transgenic studies also supported the proposed function of GPXs. For example, the overexpression of GPX in its transgenic tomato resulted in higher tolerance against abiotic stress (Herbette et al., 2011). In addition to stress response, GPXs are also thought to regulate cellular redox homeostasis by modulating the thiol-disulfide balance (Bela et al., 2015). GPX expression was found to be highly upregulated to maintain redox homeostasis under oxidative stress which helped *Brassica rapa* to adapt long-term spaceflight (Sugimoto et al., 2014).

A scan of contemporary literature reveals a paucity of information on the identification and comparative analysis of GPX and APX in model and economically important food crops. Given the above, employing bioinformatics approaches, efforts were made in this study (a) to identify potential GPX and APX genes/proteins from 18 different plant species, (b) to analyze their exon/intron organization, (c) to provide detailed information about their physico-chemical properties, (d) to identify conserved motif signatures of GPX and APX, (e) to construct their phylogenetic trees and 3D models, (f) to generate protein-protein interaction networks, and finally (g) to analyze GPX and APX gene expression profiles.

MATERIALS AND METHODS

Retrieval of GPX and APX Genes/Proteins

Eight *Arabidopsis* GPX reference protein sequences such as GPX1 (P52032.2), GPX2 (O04922.1), GPX3 (O22850.1), GPX4 (Q8L910.1), GPX5 (Q9LYB4.1), GPX6 (O48646.2), GPX7 (Q9SZ54.2), and GPX8 (Q8LBU2.1), as well as and eight *Arabidopsis* APX reference sequences such as APX1 (Q05431.2), APX2 (Q1PER6.3), APX3 (Q42564.1), APX4 (P82281.2), APX5 (Q7XZP5.2), APX6 (Q8GY91.1), APXT (Q42593.2), and APXS (Q42592.2) were obtained from UniProtKB/Swiss-Prot database of NCBI (Romiti, 2006). These reference sequences were queried in proteome datasets of selected 18 plant species: *Arabidopsis thaliana* (L.) Heynh., *Brachypodium distachyon* (L.) P. Beauv., *Brasica rapa* L., *Chlamydomonas reinhardtii* P. A. Dang., *Cucumis sativus* L., *Eucalyptus grandis* W. Hill ex Maiden, *Glycine max* (L.) Merr., *Gossypium raimondii* Ulbr., *Medicago truncatula* Gaertn., *Oryza sativa* L., *Phaseolus vulgaris* L., *Physcomitrella patens* (Hedw.) Bruch & Schimp., *Populus trichocarpa* Torr. & A.Gray ex. Hook., *Prunus persica* (L.) Batsch, *Solanum lycopersicum* L., *Sorghum bicolor* (L.) Moench, *Vitis vinifera* L., and *Zea mays* L., all found in the Phytozome v.10.3 database (Goodstein et al., 2012). After sequences were obtained, the Hidden Markov Model (HMM) search of protein sequences were performed by Pfam (http://pfam.sanger.ac.uk) to confirm the protein domain families (Finn et al., 2016). Species were arbitrarily selected to represent the main plant groups such as monocots, dicots, and lower plants.

Analysis of GPX and APX Genes/Proteins

Physicochemical properties of GPX and APX proteins were determined by using ProtParam tool (Gasteiger et al., 2005). Sub-cellular localization was predicted by CELLO (Yu et al., 2006) and WoLF PSORT (Horton et al., 2007) servers. Exon-intron organization of *GPX/APX* genes was analyzed by using a GSDS server (Hu et al., 2014). The Conserved motif structure of GPX/APX sequences was analyzed using the MEME tool with the following parameter settings: maximum number of motifs to find, 5; minimum width of motif, 6 and maximum width of motif, 50 (Bailey et al., 2009). Protein sequences were aligned by ClustalW (Thompson et al., 1994) and phylogenies were constructed by MEGA 6 (Tamura et al., 2013) with the maximum likelihood (ML) method for 1.000 bootstraps. The gene duplication events were detected using the following criteria: (a) length of alignable sequence covers >75% of the longer gene, and (b) similarity of aligned regions is >75% (Gu et al., 2002). The expression data of APX and GPX genes at anatomical and developmental levels were retrieved from the Genevestigator database (Hruz et al., 2008). 3D models of APX/GPXs were predicted by using the Phyre^2 server (Kelley and Sternberg, 2009). Model validation was performed by Rampage Ramachandran plot analysis (Lovell et al., 2003). 3D structure comparisons were done by calculating RMSD values of models using the CLICK server employing α-carbon superposition (Nguyen et al., 2011). Putative interaction partners of APX/GPXs were predicted with the STRING server (Franceschini et al., 2013) and an interactome network was generated using cytoscape (Smoot et al., 2011).

RESULTS AND DISCUSSION

H_2O_2 plays double roles in plants and modulates various crucial metabolic processes (Petrov and Van Breusegem, 2012). However, its increased levels can cause severe damage to cell structures; hence, steady-state level of cellular H_2O_2 is required to be tightly regulated (Anjum et al., 2014, 2015; Sofo et al., 2015). GPX and APX are two major ROS-scavenging enzymes which catalyze the reduction of H_2O_2 to prevent H_2O_2-derived cellular damage. In order to understand the structural, functional as well as evolutionary aspects of GPX and APX, employing bioinformatics approaches, this study attempted to present comparative analyses of putative GPX and APX homologs identified from18 plant species.

Analysis of GPXs

Retrieval of GPX Genes/Proteins

Eight potential *Arabidopsis* GPX protein sequences, namely GPX1-8, obtained from the UniProtKB/Swiss-Prot database of NCBI were used as queries in Phytozome database to retrieve the very close homologs of GPX sequences in 18 plant species. In the selection of GPX homologs from blastp hits, very strict criteria (only the highest hit sequence) was applied to avoid the redundant sequences and alternative splices of the same gene. A total of 87 GPX sequences were identified from the protein datasets of 18 plant species. These include; 8 genes for *A. thaliana,* 4 genes for *B. distachyon,* 8 genes for *B. rapa,* 1 gene for *C. reinhardtii,* 6 genes for *C. sativus,* 5 genes for *E. grandis,* 5 genes for *G. max,* 6 genes for *G. raimondii,* 5 genes for *M. truncatula,* 5 genes for *O. sativa,* 5 genes for *P. vulgaris,* 2 genes for *P. patens,* 5 genes for *P. trichocarpa,* 5 genes for *P. persica,* 5 genes for *S. lycopersicum,* 4 genes for *S. bicolor,* 5 genes for *V. vinifera,* and 3 genes for *Z. mays* (**Table 1**). Then, genomic, transcript, CDS, and protein sequences of identified 87 GPX sequences were retrieved for further analyses.

Sequence Analysis of GPX Genes/Proteins

A total of 87 GPX homologs were identified in the protein datasets of 18 plant species using *Arabidopsis* GPX1-8 for homology search. Identified GPX homologs belonged to the GSHPx (PF00255) protein family. They encoded a polypeptide of 166–262 amino acids residues (average length 197.5) and 18.4–29.7 kDa molecular weight with 4.59–9.60 *pI* value. The sequence variations in analyzed GPXs primarily derived from the "transit peptide" residues between organelle and non-organelle related GPXs (**Table 1**). Studies of molecular cloning and sequencing in *A. thaliana* have reported that chloroplastic GPX1 and GPX7 consisted of 236 and 233 amino acids, respectively; the first 1–64 residues in GPX1 and 1–69 residues in GPX7 from N-terminal site contained the transit peptides (Mullineaux et al., 1998; Lin et al., 1999; Mayer et al., 1999). *Arabidopsis* GPX2 and GPX4 were reported to be 169 and 170 residues, respectively with cytosolic localization: thereby, they did not contain any transit peptide (Lin et al., 1999). *Arabidopsis* GPX3 and GPX6 were 206

TABLE 1 | List of H_2O_2-scavenging enzyme glutathione peroxidase (GPX) homologs from 18 plant species and their primary gene/protein features.

Species name	Phytozome gene ID	Gene/protein features of GPX sequences							
		Protein domain family[a]	Domain family description	Exon no.	Protein length	MW (KDa)	*Theor. pI*	Localization CELLO[b]	Localization WoLF PSORT[b]
Arabidopsis thaliana (L.) Heynh.	AT1G63460	GSHPx (PF00255)	Glutathione peroxidase	6	167	19.0	5.11	Cyto	Cyto
	AT2G25080	GSHPx (PF00255)	Glutathione peroxidase	6	236	26.0	9.42	Chlo/Mito	Chlo
	AT2G31570	GSHPx (PF00255)	Glutathione peroxidase	6	169	18.9	5.60	Cyto	Cyto
	AT2G43350	GSHPx (PF00255)	Glutathione peroxidase	6	206	23.2	9.24	Mito/Plas	Chlo/Mito
	AT2G48150	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.3	8.87	Cyto	Mito
	AT3G63080	GSHPx (PF00255)	Glutathione peroxidase	6	173	19.3	9.28	Extr/Chlo/Nucl	Chlo
	AT4G11600	GSHPx (PF00255)	Glutathione peroxidase	6	232	25.5	9.38	Mito/Chlo	Mito
	AT4G31870	GSHPx (PF00255)	Glutathione peroxidase	6	233	25.7	9.53	Chlo	Chlo
Brachypodium distachyon (L.) P.Beauv.	Bradi1g47140	GSHPx (PF00255)	Glutathione peroxidase	6	226	24.4	9.57	Chlo	Chlo
	Bradi1g61930	GSHPx (PF00255)	Glutathione peroxidase	6	198	22.4	7.56	Cyto	Cyto
	Bradi3g51010	GSHPx (PF00255)	Glutathione peroxidase	6	240	25.9	9.05	Chlo	Chlo
	Bradi5g18000	GSHPx (PF00255)	Glutathione peroxidase	6	168	18.4	6.31	Cyto/Chlo	Nucl/Chlo
Brasica rapa L.	Brara.B02692	GSHPx (PF00255)	Glutathione peroxidase	6	229	25.2	9.21	Mito	Chlo/Mito
	Brara.C02198	GSHPx (PF00255)	Glutathione peroxidase	6	197	21.9	8.55	Extr/Plas	Extr
	Brara.E00003	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.2	9.05	Extr/Cyto	Chlo
	Brara.E01208	GSHPx (PF00255)	Glutathione peroxidase	6	169	18.9	6.34	Cyto	Cyto
	Brara.G01994	GSHPx (PF00255)	Glutathione peroxidase	6	176	19.5	9.15	Extr/Cyto/Nucl	Chlo
	Brara.I01234	GSHPx (PF00255)	Glutathione peroxidase	6	167	18.9	5.00	Cyto	Cyto
	Brara.I04448	GSHPx (PF00255)	Glutathione peroxidase	6	233	25.8	9.29	Mito/Chlo/Extr	Chlo
	Brara.K00392	GSHPx (PF00255)	Glutathione peroxidase	6	232	25.9	9.60	Mito/Extr	Chlo
Chlamydomonas reinhardtii P.A.Dang.	Cre03.g197750	GSHPx (PF00255)	Glutathione peroxidase	7	200	21.9	9.39	Mito	Chlo
Cucumis sativus L.	Cucsa.084960	GSHPx (PF00255)	Glutathione peroxidase	6	176	19.7	8.86	Cyto	Chlo
	Cucsa.094950	GSHPx (PF00255)	Glutathione peroxidase	6	204	23.4	8.55	Plas/Extr	Chlo
	Cucsa.184280	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.0	8.66	Cyto/Extr	Cyto
	Cucsa.271420	GSHPx (PF00255)	Glutathione peroxidase	6	241	26.4	9.5	Chlo	Chlo
	Cucsa.303050	GSHPx (PF00255)	Glutathione peroxidase	6	241	26.8	9.28	Mito	Chlo/Mito
	Cucsa.303070	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.2	5.21	Cyto	Cyto
Eucalyptus grandis W. Hill ex Maiden	Eucgr.A00257	GSHPx (PF00255)	Glutathione peroxidase	6	202	22.8	7.62	Extr/Chlo	Chlo/Vacu
	Eucgr.C02602	GSHPx (PF00255)	Glutathione peroxidase	6	247	26.9	9.53	Chlo	Chlo
	Eucgr.D01856	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.4	5.16	Cyto	Cyto
	Eucgr.E00579	GSHPx (PF00255)	Glutathione peroxidase	6	250	27.3	9.16	Chlo	Chlo
	Eucgr.K03389	GSHPx (PF00255)	Glutathione peroxidase	6	170	18.9	9.02	Cyto	Chlo/Nucl
Glycine max (L.) Merr.	Glyma.03G151500	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.0	9.45	Mito/Cyto	Chlo
	Glyma.05G240100	GSHPx (PF00255)	Glutathione peroxidase	6	199	22.7	7.54	Extr	Extr
	Glyma.08G013900	GSHPx (PF00255)	Glutathione peroxidase	6	167	18.9	5.09	Cyto	Chlo
	Glyma.11G024100	GSHPx (PF00255)	Glutathione peroxidase	6	167	18.5	5.88	Cyto	Cyto
	Glyma.17G223900	GSHPx (PF00255)	Glutathione peroxidase	6	234	26.3	9.40	Mito/Chlo	Chlo
Gossypium raimondii Ulbr.	Gorai.001G038600	GSHPx (PF00255)	Glutathione peroxidase	6	242	26.6	9.30	Chlo	Chlo
	Gorai.004G083200	GSHPx (PF00255)	Glutathione peroxidase	6	171	19.1	9.24	Nucl/Cyto/Extr	Nucl
	Gorai.004G087300	GSHPx (PF00255)	Glutathione peroxidase	6	208	23.6	5.51	Extr	Extr

(Continued)

TABLE 1 | Continued

Species name	Phytozome gene ID	Gene/protein features of GPX sequences							
		Protein domain family[a]	Domain family description	Exon no.	Protein length	MW (KDa)	*Theor. pI*	Localization CELLO[b]	Localization WoLF PSORT[b]
	Gorai.004G211400	GSHPx (PF00255)	Glutathione peroxidase	6	166	18.4	6.73	Cyto	Chlo
	Gorai.006G186100	GSHPx (PF00255)	Glutathione peroxidase	6	168	18.7	6.73	Cyto	Cyto
	Gorai.008G246600	GSHPx (PF00255)	Glutathione peroxidase	6	168	19.1	4.59	Cyto	Chlo
Zea mays L.	GRMZM2G012479	GSHPx (PF00255)	Glutathione peroxidase	6	230	24.9	9.55	Mito	Chlo
	GRMZM2G144153	GSHPx (PF00255)	Glutathione peroxidase	6	168	18.4	6.58	Cyto	Chlo/Nucl
	GRMZM2G329144	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.2	7.58	Cyto	Chlo
Vitis vinifera L.	GSVIVG01010737001	GSHPx (PF00255)	Glutathione peroxidase	6	167	18.6	5.53	Cyto	Cyto
	GSVIVG01011101001	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.0	9.22	Cyto	Mito
	GSVIVG01019765001	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.2	5.01	Cyto	Cyto
	GSVIVG01019766001	GSHPx (PF00255)	Glutathione peroxidase	6	168	18.6	6.73	Cyto	Chlo/Extr
	GSVIVG01035981001	GSHPx (PF00255)	Glutathione peroxidase	6	207	22.9	9.16	Chlo/Mito	Chlo
Oryza sativa L.	LOC_Os02g44500	GSHPx (PF00255)	Glutathione peroxidase	6	238	25.8	9.42	Chlo	Chlo
	LOC_Os03g24380	GSHPx (PF00255)	Glutathione peroxidase	6	169	19.2	8.80	Cyto	Cyto
	LOC_Os04g46960	GSHPx (PF00255)	Glutathione peroxidase	6	168	18.4	8.33	Cyto	Chlo
	LOC_Os06g08670	GSHPx (PF00255)	Glutathione peroxidase	6	234	25	9.51	Mito/Chlo	Chlo
	LOC_Os11g18170	GSHPx (PF00255)	Glutathione peroxidase	6	212	22.9	7.62	Chlo/Extr	Chlo
Medicago truncatula Gaertn.	Medtr1g014210	GSHPx (PF00255)	Glutathione peroxidase	6	236	26.4	9.32	Mito/Chlo	Chlo
	Medtr7g094600	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.2	9.18	Cyto/Mito	Nucl
	Medtr8g098400	GSHPx (PF00255)	Glutathione peroxidase	6	172	19.3	4.82	Cyto	Chlo
	Medtr8g098410	GSHPx (PF00255)	Glutathione peroxidase	6	233	25.8	9.27	Mito	Chlo
	Medtr8g105630	GSHPx (PF00255)	Glutathione peroxidase	6	167	18.9	8.32	Plas	Chlo
Physcomitrella patens (Hedw.) Bruch & Schimp.	Phpat.004G103100	GSHPx (PF00255)	Glutathione peroxidase	6	247	26.7	9.24	Chlo/Extr	Chlo
	Phpat.017G045400	GSHPx (PF00255)	Glutathione peroxidase	1	170	19.1	8.30	Cyto	Cyto
Phaseolus vulgaris L.	Phvul.001G041100	GSHPx (PF00255)	Glutathione peroxidase	6	262	29.7	9.68	Mito	Chlo
	Phvul.001G149000	GSHPx (PF00255)	Glutathione peroxidase	6	168	18.8	9.31	Cyto/Nucl	Nucl
	Phvul.002G157200	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.0	4.97	Cyto	Chlo/Nucl
	Phvul.002G288700	GSHPx (PF00255)	Glutathione peroxidase	6	230	25.6	8.76	Chlo/Mito	Chlo
	Phvul.002G322400	GSHPx (PF00255)	Glutathione peroxidase	6	198	22.5	5.94	Extr	Extr
Populus trichocarpa Torr. & A.Gray ex. Hook.	Potri.001G105100	GSHPx (PF00255)	Glutathione peroxidase	5	170	19.3	4.78	Cyto	Cyto
	Potri.003G126100	GSHPx (PF00255)	Glutathione peroxidase	6	238	26.2	9.29	Mito/Chlo	Chlo
	Potri.006G265400	GSHPx (PF00255)	Glutathione peroxidase	6	232	25.3	9.48	Chlo/Mito	Chlo
	Potri.007G126600	GSHPx (PF00255)	Glutathione peroxidase	6	203	22.8	6.83	Extr	Extr/Vacu
	Potri.014G138800	GSHPx (PF00255)	Glutathione peroxidase	6	170	18.9	9.15	Cyto/Extr	Chlo/Cyto
Prunus persica (L.) Batsch	ppa010584m.g	GSHPx (PF00255)	Glutathione peroxidase	6	244	26.7	9.33	Chlo	Chlo
	ppa010771m.g	GSHPx (PF00255)	Glutathione peroxidase	6	237	25.9	9.20	Mito	Chlo
	ppa011682m.g	GSHPx (PF00255)	Glutathione peroxidase	6	200	22.7	8.27	Extr/Cyto	Extr
	ppa012378m.g	GSHPx (PF00255)	Glutathione peroxidase	6	172	19.4	8.97	Cyto	Nucl/Cyto
	ppa012416m.g	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.4	4.86	Cyto	Chlo
Sorghum bicolor (L.) Moench	Sobic.001G365800	GSHPx (PF00255)	Glutathione peroxidase	6	171	19.3	8.79	Cyto	Chlo
	Sobic.006G173900	GSHPx (PF00255)	Glutathione peroxidase	6	168	18.4	6.58	Cyto	Chlo/Nucl

(Continued)

TABLE 1 | Continued

Species name	Phytozome gene ID	Gene/protein features of GPX sequences							
		Protein domain family[a]	Domain family description	Exon no.	Protein length	MW (KDa)	*Theor. pI*	Localization CELLO[b]	Localization WoLF PSORT[b]
	Sobic.010G067100	GSHPx (PF00255)	Glutathione peroxidase	6	232	24.9	9.50	Mito/Chlo	Chlo
	Sobic.K022000	GSHPx (PF00255)	Glutathione peroxidase	6	205	22.6	5.68	Cyto/Extr	Mito/Chlo
Solanum lycopersicum L.	Solyc06g073460.2	GSHPx (PF00255)	Glutathione peroxidase	6	167	18.9	6.37	Cyto	Chlo
	Solyc08g006720.2	GSHPx (PF00255)	Glutathione peroxidase	6	238	26.2	9.18	Chlo	Chlo
	Solyc08g080940.2	GSHPx (PF00255)	Glutathione peroxidase	6	239	26.7	9.16	Mito	Chlo
	Solyc09g064850.2	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.0	9.33	Mito/Extr	Chlo
	Solyc12g056240.1	GSHPx (PF00255)	Glutathione peroxidase	6	170	19.3	4.97	Cyto	Cyto

[a] *Protein domain families were searched in Pfam database.*

[b] *Cyto, Cytosolic; Chlo, Chloroplastic; Mito, Mitochondrial; Vacu, Vacuolar; Nucl, Nuclear; Extr, Extracellular; Plas, Plasma membrane.*

More than one localization specified in a single column also shows the significance of other entries in order.

and 232 residues, respectively, with mitochondrial localizations; the first 1–12 amino acids in GPX3 and 1–54 residues in GPX6 covered the transit peptide (Lin et al., 1999; Mayer et al., 1999). *Arabidopsis* GPX5 was 173 residues with probable ER or Plasma membrane localization, without transit peptide (Erfle et al., 2000). *Arabidopsis* GPX8 comprised of 167 amino acids with cytosolic or nuclear localization, without transit peptide (Theologis et al., 2000). In the present study, alignment analysis revealed that in chloroplastic/mitochondrial-related GPXs, the transit peptide sequences formed the first 50–90 amino acid residues from the N-terminal site while in extra cellular/plasma membrane-related GPXs, residues of the first 20–50 amino acid from N-terminal region contained the putative transit peptide. However, cytosolic sequences lacked of any putative transit residues (Supplementary Figure S1). Thus, analyzed GPX sequences were roughly categorized in three main groups based on their sequence length; the chloroplastic/mitochondrial related GPXs comprised the longer sequences (i), extra cellular/plasma membrane related GPXs formed the medium-sized sequences (ii), and cytosolic related GPXs included the shorter sequences (iii). In addition, the regions corresponding to the transit peptide sites in analyzed sequences did not demonstrate any particular patterns. The less-conserved transit peptide residues could be related with the functional diversities of GPXs at various targets. However, despite the variations in sequence length and transit peptide residues, transcripts of GPX homologs mainly contained the six exons. Therefore, it is reasonable to claim that analyzed GPX sequences could have highly-conserved protein sequences, preserved during the formation of various GPXs. The alignment analysis of 87 GPX protein homologs also confirmed this claim, demonstrating the presence of more conserved residues in the main sites of the active enzyme (Supplementary Figure S2). Moreover, to discern the conserved motif patterns in GPX sequences, the most conserved five motif sequences were searched in sets of 87 GPX homologs using MEME tool (**Table 2**). Motif 1 and 3 were the 50 amino acid residues, while the motif 2 was 41, motif 4 was 15, and motif 5 was 6 residues in length. Motif 1 and 3 were related with the GSHPx (PF00255) protein family and present in almost all GPX homologs. The presence of long conserved residues and their relation with the GSHPx family could indicate the highly conserved structures of GPX sequences at these sites between/among species.

Furthermore, alignment analysis also demonstrated that Asn (N), Gly (G), Arg (R), Pro (P), Thr (T), Tyr (Y), Lys (K), Ala-Ser (AS), Cys-Gly (CG), Phe-Pro (FP), Glu-Pro (EP), Leu-Lys (LK), Lys-Phe (KF), Asn-Gly (NG), Asn-Gln-Phe (NQF), and Trp-Asn-Phe (WNF) residues were strictly conserved in all aligned sequences, indicating their potential functions in enzyme activity and/or stability (Supplementary Figure S3). To infer a functional relationship between these conserved residues and GPX sequences, we searched for the known catalytic residues of model organism *Arabidopsis* GPXs in the UniProtKB/Swiss-Prot database of NCBI (www.ncbi.nlm.nih.gov/protein). The following residues were designated in the database as potential catalytic residues for *Arabidopsis* GPX1-8: GPX1 (Cys-111, Gln-146, Trp-200), GPX2 (Cys-41, Gln-76, Trp-130), GPX3 (Cys-80, Gln-115, Trp-169), GPX4 (Cys-44, Gln-79, Trp-133), GPX5 (Cys-46, Gln-81, Trp-135), GPX6 (Cys-105, Gln-140, Trp-194), GPX7 (Cys-108, Gln-143, Trp-197), and GPX8 (Cys-41, Glu-76, Trp-130). Interestingly, residues that correspond to these catalytic sites in other analyzed sequences were found to be strictly conserved (Supplementary Figure S3). This shows that active sites of the enzyme are considerably conserved between species.

Phylogenetic Analysis of GPXs

The evolutionary relationships between identified GPX sequences were analyzed by MEGA 6 using the Maximum Likelihood (ML) method with 1000 bootstraps. The constructed phylogeny included all 87 GPX homologs to discover the phylogenetic distribution of sequences (**Figure 1**). The tree was divided into six major groups based on the tree topology, and each group was indicated with a different color segment. The red segment included cytosolic, extra cellular, and plasma membrane

TABLE 2 | Most conserved five motifs of glutathione peroxidase (GPX) homologs in 18 plant species.

Motif	Width	Identified site no.	Sequence	Protein domain family[a]
1	50	87 of 87	KYKDQGFEILAFPCNQFGGQEPGTNEEIQQFACTRFKAEYPIFDKVDVNG	GSHPx (PF00255)
2	41	87 of 87	FGDRIKWNFTKFLVDKEGHVVDRYAPTTSPLQIEKDIQKLL	Not found
3	50	86 of 87	KSIHDFTVKDIRGNDVDLSIYKGKVLLIVNVASQCGMTNSNYTELNHLYE	GSHPx (PF00255)
4	15	87 of 87	NAAPLYKFLKSSKWG	Not found
5	6	63 of 87	MAASHS	Not found

[a] *Protein domain families have been searched in Pfam database.*

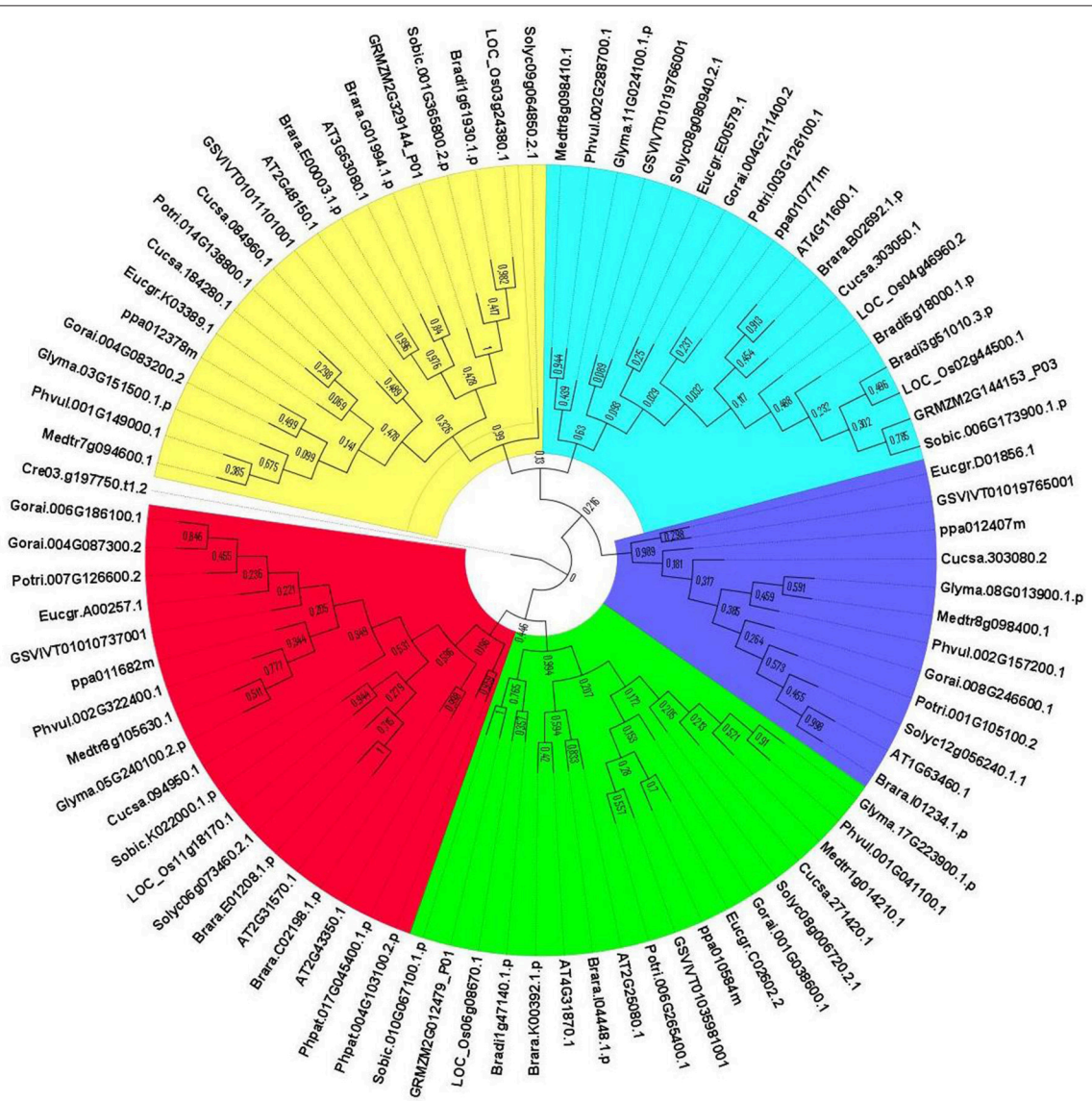

FIGURE 1 | Phylogenetic tree of glutathione peroxidase (GPX) homologs from 18 plant species. Tree was constructed by MEGA 6 using Maximum likelihood (ML) method with 1000 bootstraps. Segment classification based on the consensus of two subcellular localization servers, CELLO and WoLF PSORT as well as tree topology for ambiguous sequences. Red segment includes cytosolic, extra cellular, and plasma membrane related GPXs, green segment contains mitochondrial and chloroplast related GPXs, blue segment only have cytosolic GPXs, cyan segment includes cytosolic, and chloroplast/mitochondrial related GPXs, yellow segment contains cytosolic/nuclear related GPXs, and non-colored segment has lower plant Chlamydomonas GPX with chloroplastic/mitochondrial relation.

related GPXs, the green segment contained mitochondrial and chloroplast related GPXs, the blue segment only had cytosolic GPXs, the cyan segment included cytosolic and chloroplast/mitochondrial related GPXs, the yellow segment contained cytosolic/nuclear related GPXs, and the non-colored segment had lower plant *Chlamydomonas* GPX with a chloroplastic/mitochondrial relation. Annotation of each segment based on the consensus of two subcellular localization servers, CELLO and WoLF PSORT, as well as tree topology for ambiguous sequences. Mainly cytosolic, nuclear, extra cellular and plasma membrane related GPXs were clustered together, while chloroplast/mitochondrial related GPXs also cluster together. Therefore, the presence or absence of transit peptide residues was the main contributing entity in the phylogenetic distribution of GPX sequences. In addition, the presence of sequences with different subcellular localizations in the same group inferred the possibility of gene duplication events in the formation of various GPX sequences. Duplications in plant genomes could be either as small-scale such as tandem and segmental duplications, or as large-scale such as whole-genome duplications (Ramsey and Schemske, 1998). The segmental duplications are observed in different chromosomes whereas tandem duplications occur in the same chromosome (Liu et al., 2011). Several segmental duplications were identified between GPX pairs (**Table 3**). The presence of segmental duplications, particularly between sequences with various subcellular localizations may partly explain the possibility of gene duplication events in GPX formations.

TABLE 3 | The segmental duplication events in some glutathione peroxidase (GPX) pairs.

Species name	Segmental duplication pairs
Arabidopsis thaliana (L.) Heynh.	AT2G25080-AT4G31870
	AT2G48150-AT3G63080
Brachypodium distachyon (L.) P.Beauv.	Bradi5g18000-Bradi3g51010
Brasica rapa L.	Brara.E00003-Brara.G01994
	Brara.I04448-Brara.K00392
Gossypium raimondii Ulbr.	Gorai.004G087300-Gorai.006G186100
	Gorai.004G211400-Gorai.008G246600
Vitis vinifera L.	GSVIVG01019765001-GSVIVG01019766001
Oryza sativa L.	LOC_Os04g46960-LOC_Os02g44500
Physcomitrella patens (Hedw.) Bruch & Schimp.	Phpat.017G045400-Phpat.004G103100
Prunus persica (L.) Batsch	ppa012416m.g-ppa010771m.g

Expression Profile Analysis of GPXs

The potential expression profile of *GPX* genes was analyzed at 105 anatomical parts and 10 developmental stage levels using model organism *A. thaliana* GPXs from Genevestigator platform (**Figure 2**). Eight *Arabidopsis* genes, namely GPX1 (AT2G25080), GPX2 (AT2G31570), GPX3 (AT2G43350), GPX4 (AT2G48150), GPX5 (AT3G63080), GPX6 (AT4G11600), GPX7 (AT4G31870), and GPX8 (AT1G63460) were retrieved from the "Affymetrix Arabidopsis ATH1 Genome Array" platform using the Genevestigator interface, and conditions and genes with similar profiles were comparatively analyzed using the Hierarchical clustering tool with the Euclidean distance method. At an anatomical part level (**Figure 2A**), analyzed *GPX1-8* genes were expressed in almost all 105 anatomical tissues in *Arabidopsis* plants. However, various root and leaf protoplast cells, seed-related tissues, and active growth zones demonstrated significantly higher GPX activity. This indicates that stress factors and/or active metabolism could lead to the up-regulation of various *GPX* genes in different tissues. Many studies have also showed that balance between production and scavenging of H_2O_2 could be disturbed by various biotic/abiotic stress factors or perturbations such as drought, salinity, pathogen attack, oxidative state of the cells (Apel and Hirt, 2004; Anjum et al., 2014, 2015; Sofo et al., 2015). Besides, a number of studies were also available demonstrating the functional roles of GPXs in plant stress resistance/tolerance. For example, a *GPX* gene in *Pennisetum glaucum* enhanced the drought and salinity stress tolerance (Islam et al., 2015). Citrus *GPX3* was reported to be essential in detoxification of ROS-induced cellular stresses as well as in *Alternaria alternata* pathogenesis (Yang et al., 2016). Silencing of mitochondrial *GPX1* in *O. sativa* demonstrated the impaired photosynthesis in response to salinity (Lima-Melo et al., 2016). Glutathione transferases and peroxidases were reported as key components in *Arabidopsis* salt stress-acclimation (Horváth et al., 2015). *GPX1* and *GPX3* in legume root nodules played a protective function against salt stress, oxidative stress, and membrane damage (Matamoros et al., 2015). Therefore, GPXs, which are the antioxidant enzymatic components of the cells, are consequently induced to suppress/eliminate the toxic levels of H_2O_2. The increased GPX activities in analyzed *Arabidopsis* tissues could thereby be derived from the increased H_2O_2 or H_2O_2-related products. In addition, clustering analysis of *GPX* genes showed that *GPX2, 3, 5, 6,* and *8* demonstrate relatively similar expression profiles compared to those of *GPX1, 4,* and *7*. At the developmental level (**Figure 2B**), the expression profiles of *Arabidopsis GPX1-8* genes were analyzed at 10 developmental stages, including senescence, mature siliques, flowers and siliques, developed flower, young rosette, germinated seed, seedling, bolting, young flower, and developed rosette. *GPX1-8* were relatively expressed in all developmental stages. However, the expression of *GPX*s in the senescence stage demonstrated slightly different patterns, particularly the mitochondrial *GPX6* gene had the highest expression profile compared to other developmental stages. This may have been caused by senescence-related cellular deteriorations, leading to the substantial metabolic or physiological changes that significantly affect the overall metabolic energy consumption. Therefore, it seems that the expression profiles of *GPX*s are highly associated with the metabolic state of the cells.

3D Structure Analysis of GPXs

3D models of putative GPXs were constructed by using Phyre^2 server for eight identified *Arabidopsis GPX1-8* gene sequences (**Figure 3**). These sequences were: AT2G25080.1 (*GPX1*), AT2G31570.1 (*GPX2*), AT2G43350.1 (*GPX3*), AT2G48150.1 (*GPX4*), AT3G63080.1 (*GPX5*), AT4G11600.1

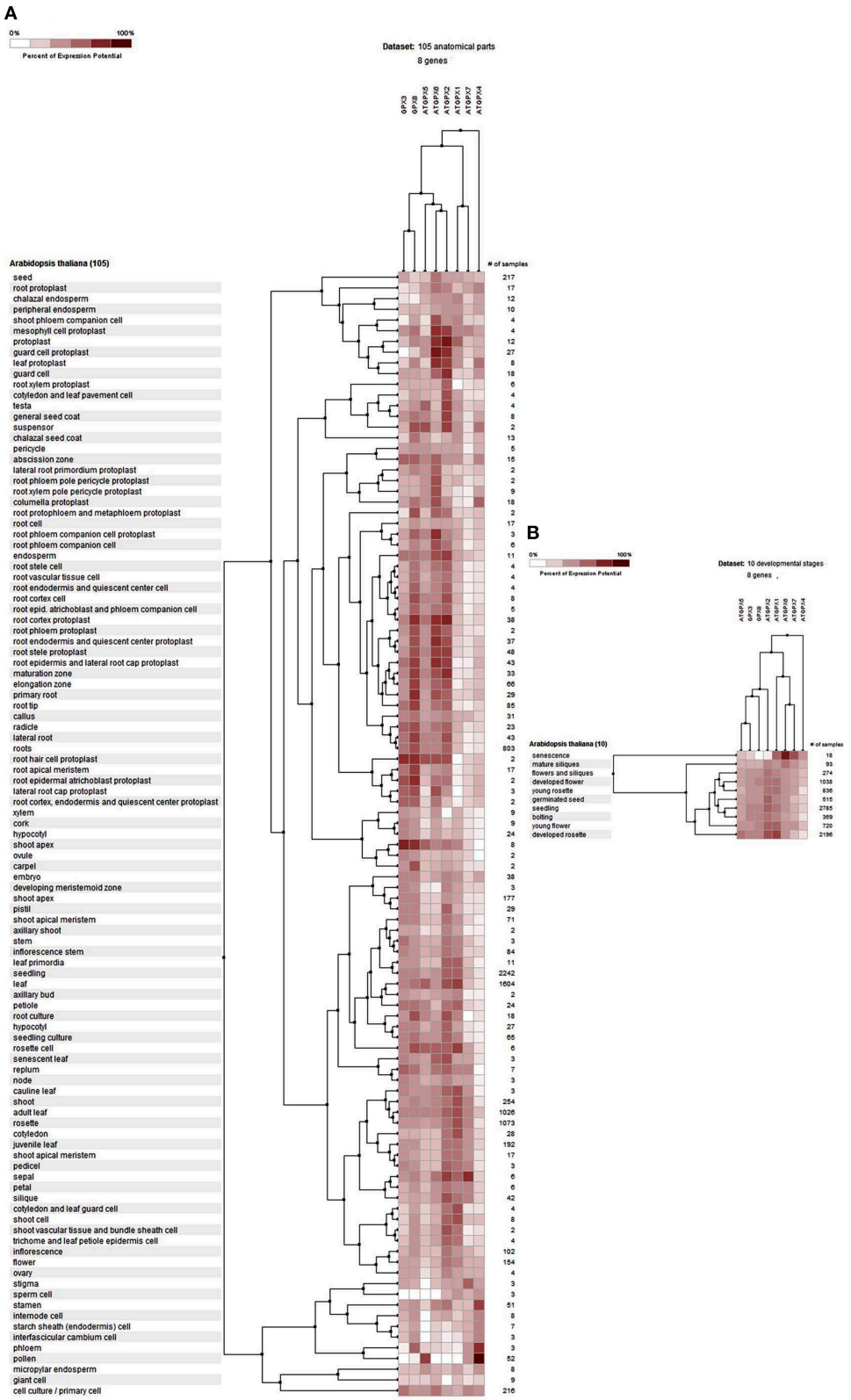

FIGURE 2 | Expression profile of *Arabidopsis* glutathione peroxidase *GPX1-8* genes at 105 anatomical parts (A) and 10 developmental stage levels (B). Genes and conditions with similar profiles were comparatively analyzed using hierarchical clustering tool with Euclidean distance method at Genevestigator platform.

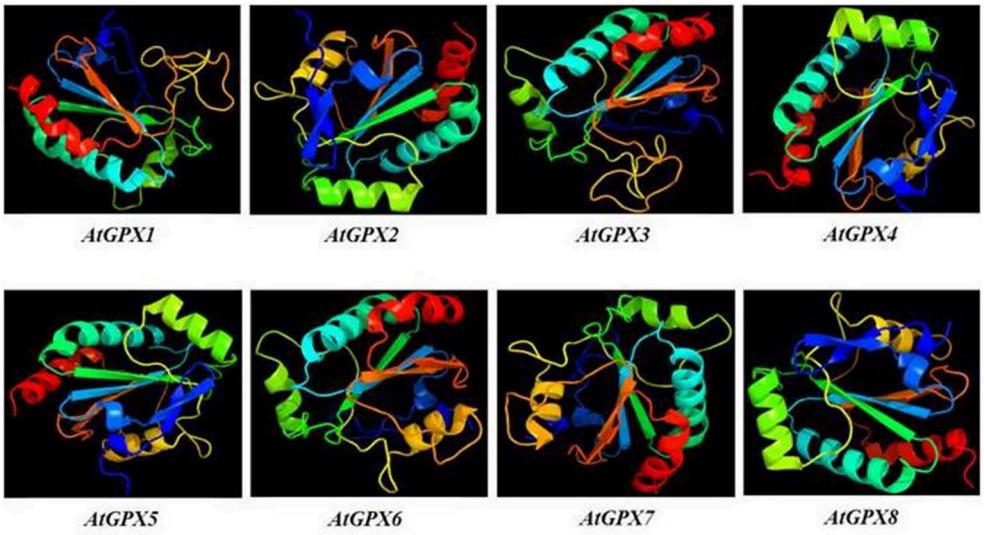

FIGURE 3 | 3D models of predicted *Arabidopsis* glutathione peroxidase GPX1-8 sequences. Models were constructed by using Phyre^2 server for AT2G25080.1 (GPX1), AT2G31570.1 (GPX2), AT2G43350.1 (GPX3), AT2G48150.1 (GPX4), AT3G63080.1 (GPX5), AT4G11600.1 (GPX6), AT4G31870.1 (GPX7), and AT1G63460.1 (GPX8) sequences, and colored by rainbow from N- to C-terminus.

(*GPX6*), AT4G31870.1 (*GPX7*), and AT1G63460.1 (*GPX8*). In modeling, three templates such as 2F8A:A (*GPX1*, *GPX3*, *GPX6,* and *GPX7*), 2V1M:A (*GPX2* and *GPX5*), and 2P5Q:A (*GPX4* and *GPX8*) were used to maximize the alignment coverage, percentage identity and confidence for submitted sequences. Predicted models demonstrated the ≥98% of residues in allowed region in Ramachandran plot analysis, indicating that constructed models were fairly in good quality. To find out the structural divergence/similarity in generated models, the structures were superposed to calculate the percentage of structural overlap and RMSD values (**Table 4**). GPX1-GPX3, GPX4-GPX8, and GPX6-GPX7 pairs demonstrated the highly conserved structural overlap (100%) with 0.14, 0.00, and 0.03 RMSD values, respectively. The each designated pair also belonged to either chloroplastic/mitochondrial or cytosolic form, indicating their functional similarities with minor specifications. In addition, GPX1-GPX6 and 7, GPX2-GPX5, and GPX3-GPX6 and 7 pairs showed very high structural similarity with ≥94 structural overlaps. Despite the highly conserved structures of *Arabidopsis* GPX members, some minor variations were also present. It seems that these divergences in GPXs may not change the protein-3D structure, however, they could attribute the new functional roles to catalytic activities.

Interaction Partner Analysis of GPXs

The interactome network was constructed for 10 putative interactors of *Arabidopsis* cytosolic GPX2, using Cytoscape with STRING data (**Figure 4**). APX1 (L-ascorbate peroxidase), GSH2 (glutathione synthetase), GSTF6 (glutathione S-transferase F6), GSTT1 (glutathione S-transferase THETA 1), PER1 (1-Cys peroxiredoxin PER1), AT1G65820 (glutathione S-transferase), GSTF12 (glutathione S-transferase phi 12), GSTF2 (glutathione S-transferase F2), GSTF8 (glutathione S-transferase F8), and GSTU19 (glutathione S-transferase U19) proteins were predicted as the main interaction partners of *Arabidopsis* cytosolic GPX2. APX1 is a type of H_2O_2-scavenging enzyme and a central component in the reactive oxygen gene network (Storozhenko et al., 1998; Fourcroy et al., 2004). GSH2 involves in the second step of the glutathione synthesis pathway from L-cysteine and L-glutamate (Wang and Oliver, 1996). GSTF6 functions in camalexin biosynthesis, is involved in the conjugation of reduced glutathione to various exogenous/endogenous hydrophobic electrophiles, and has a detoxification role for certain herbicides (Su et al., 2011). GSTT1, GSTF8, and GSTU19 are reported to have glutathione S-transferase or peroxidase activity. They further conjugate the reduced glutathione to various exogenous/endogenous hydrophobic electrophiles and play a detoxification role for certain herbicides (Wagner et al., 2002). PER1 is an antioxidant protein involved in the inhibition of germination under stress (Haslekås et al., 1998). AT1G65820 is a glutathione S-transferase. GSTF12 is involved in the transport of anthocyanins and proanthocyanidins into the vacuole (Kitamura et al., 2004). GSTF2 plays a role in binding and transport of small bioactive products and defense-related compounds under stress (Smith et al., 2003). The analysis indicated that cytosolic GPX2 enzyme is closely related with various pathways involving in antioxidant and secondary metabolite metabolisms, thereby supporting the functional role of GPXs in H_2O_2-scavenging and plant defense.

Analysis of APXs

Retrieval of APX Genes/Proteins

Eight potential *Arabidopsis* APX protein sequences such as APX1-6, APXT, and APXS, obtained from the UniProtKB / Swiss-Prot database of NCBI, were used as queries in Phytozome database to retrieve the very close homologs of APX sequences in

TABLE 4 | Structural overlap (%)/RMSD values in superposed *Arabidopsis* glutathione peroxidases (GPXs).

	GPX1	GPX2	GPX3	GPX4	GPX5	GPX6	GPX7	GPX8
GPX1	–	88.68/1.03	100.00/0.14	91.19/1.10	89.94/0.91	94.34/0.24	94.34/0.24	91.19/1.10
GPX2	88.05/0.89	–	88.68/0.93	93.75/0.66	99.38/0.15	91.82/0.92	91.82/0.92	94.38/0.71
GPX3	100.00/0.14	89.94/1.10	–	90.57/0.90	90.57/0.95	94.34/0.28	94.34/0.28	90.57/0.90
GPX4	89.94/1.07	93.75/0.67	91.19/0.96	–	95.65/0.67	94.97/0.97	94.97/0.97	100.00/0.00
GPX5	90.57/0.89	99.38/0.15	90.57/0.91	95.65/0.66	–	94.34/0.91	94.34/0.91	95.65/0.67
GPX6	94.34/0.24	92.45/1.04	94.34/0.28	95.60/1.07	94.97/1.04	–	100.00/0.03	95.60/1.06
GPX7	94.34/0.24	92.45/1.05	94.34/0.28	95.60/1.06	94.97/1.04	100.00/0.03	–	95.60/1.06
GPX8	91.19/0.99	95.00/0.75	91.19/0.98	100.00/0.00	95.65/0.67	94.97/0.97	94.97/0.97	–

Red-color highlighted pairs show the highly conserved structural overlaps.

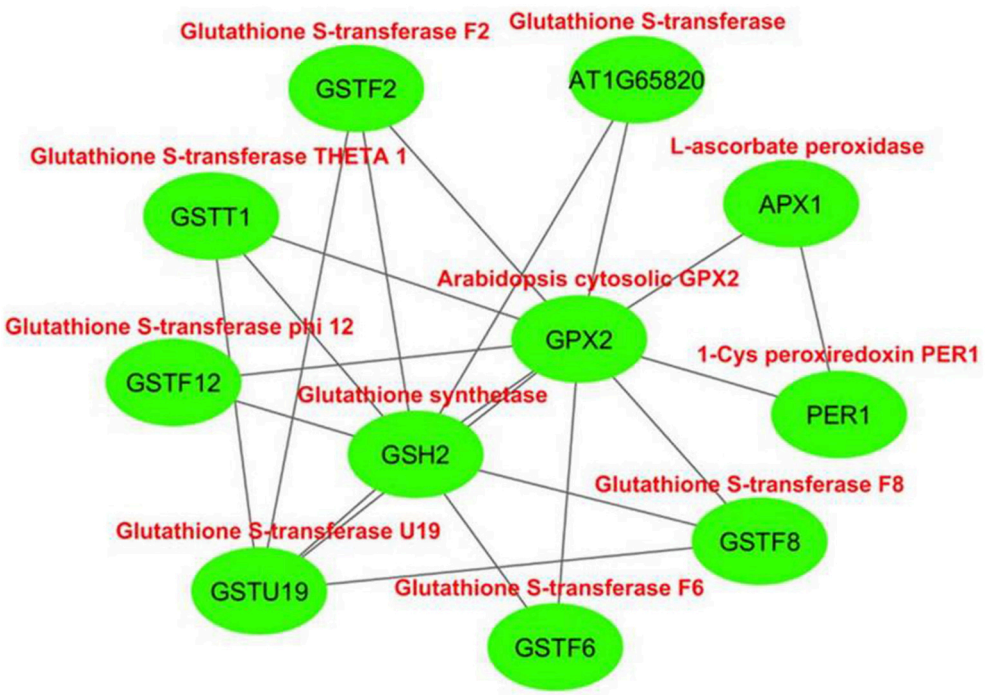

FIGURE 4 | Predicted 10 interaction partners of *Arabidopsis* cytosolic glutathione peroxidase GPX2. Interactome was generated using cytoscape with STRING data. APX1 (L-ascorbate peroxidase), GSH2 (glutathione synthetase), GSTF6 (glutathione S-transferase F6), GSTT1 (glutathione S-transferase THETA 1), PER1 (1-Cys peroxiredoxin PER1), AT1G65820 (glutathione S-transferase), GSTF12 (glutathione S-transferase phi 12), GSTF2 (glutathione S-transferase F2), GSTF8 (glutathione S-transferase F8), and GSTU19 (glutathione S-transferase U19) proteins were predicted as main interaction partners of *Arabidopsis* cytosolic GPX2.

18 plant species. In the selection of APX homologs from blastp hits, a very strict criterion (only the highest hit sequence) was applied to avoid redundant sequences and alternative splices of the same gene. A total of 120 APX sequences were identified from the protein datasets of 18 plant species. These were 8 genes for *A. thaliana,* 7 genes for *B. distachyon,* 8 genes for *B. rapa,* 4 genes for *C. reinhardtii,* 5 genes for *C. sativus,* 7 genes for *E. grandis,* 7 genes for *G. max,* 8 genes for *G. raimondii,* 7 genes for *M. truncatula,* 6 genes for *O. sativa,* 6 genes for *P. vulgaris,* 5 genes for *P. patens,* 7 genes for *P. trichocarpa,* 6 genes for *P. persica,* 7 genes for *S. lycopersicum,* 8 genes for *S. bicolor,* 6 genes for *V. vinifera,* and 8 genes for *Z. mays* (**Table 5**). Then, genomic, transcript, CDS, and protein sequences of 120 identified APX sequences were retrieved for further analyses.

Sequence Analysis of APX Genes/Proteins

A total of 120 APX homologs were identified in protein datasets of 18 plant species using *Arabidopsis* APX1-6, APXT, and APXS sequences by homology search. Identified APX sequences contained the peroxidase (PF00141) protein family domain. They encoded a protein of 197–478 amino acids residues (average length 323.9) and 23.7–52.1 kDa molecular weight with 5.03–9.23 *pI* value. The sequence variations in analyzed APXs demonstrated a correlation with their putative localizations,

TABLE 5 | List of H_2O_2-scavenging enzyme ascorbate peroxidase (APX) homologs from 18 plant species and their primary gene/protein features.

Species name	Phytozome gene ID	Gene/protein features of GPX sequences							
		Protein domain family[a]	Domain family description	Exon no.	Protein length	MW (KDa)	*Theor. pI*	Localization CELLO[b]	Localization WoLF PSORT[b]
Arabidopsis thaliana (L.) Heynh.	AT1G07890	Peroxidase (PF00141)	Peroxidase	8	250	27.5	5.72	Cyto	Cyto
	AT1G77490	Peroxidase (PF00141)	Peroxidase	12	426	46.0	6.81	Chlo	Chlo
	AT3G09640	Peroxidase (PF00141)	Peroxidase	9	251	28.0	5.87	Cyto	Cyto
	AT4G08390	Peroxidase (PF00141)	Peroxidase	10	372	40.4	8.31	Chlo	Chlo
	AT4G09010	Peroxidase (PF00141)	Peroxidase	10	349	37.9	8.59	Chlo/Mito	Chlo
	AT4G32320	Peroxidase (PF00141)	Peroxidase	10	329	36.2	8.99	Chlo	Chlo
	AT4G35000	Peroxidase (PF00141)	Peroxidase	9	287	31.5	6.47	Cyto	Cyto
	AT4G35970	Peroxidase (PF00141)	Peroxidase	9	279	30.8	8.80	Cyto/Nucl	Cyto
Brachypodium distachyon (L.) P.Beauv.	Bradi1g16510	Peroxidase (PF00141)	Peroxidase	9	256	27.7	5.28	Cyto	Cyto
	Bradi1g65820	Peroxidase (PF00141)	Peroxidase	9	250	27.4	5.71	Cyto	Cyto
	Bradi3g40330	Peroxidase (PF00141)	Peroxidase	11	329	35.4	6.36	Chlo	Chlo
	Bradi3g42340	Peroxidase (PF00141)	Peroxidase	9	289	31.5	7.70	Cyto/Chlo	Cyto
	Bradi3g45700	Peroxidase (PF00141)	Peroxidase	12	439	47.3	5.61	Chlo	Chlo
	Bradi5g10490	Peroxidase (PF00141)	Peroxidase	11	345	37.4	8.77	Chlo/Mito	Chlo
	Bradi5g20670	Peroxidase (PF00141)	Peroxidase	10	333	36.1	8.71	Mito	Chlo
Brasica rapa L.	Brara.A00250	Peroxidase (PF00141)	Peroxidase	8	280	31.0	7.69	Cyto	Cyto
	Brara.A03521	Peroxidase (PF00141)	Peroxidase	9	251	28.1	6.41	Cyto	Cyto
	Brara.C02583	Peroxidase (PF00141)	Peroxidase	9	348	37.9	8.59	Chlo/Mito	Chlo
	Brara.G03518	Peroxidase (PF00141)	Peroxidase	10	439	47.5	7.70	Chlo	Chlo
	Brara.I02406	Peroxidase (PF00141)	Peroxidase	10	354	38.8	7.12	Chlo/Mito	Chlo
	Brara.I05334	Peroxidase (PF00141)	Peroxidase	7	250	27.5	5.61	Cyto	Cyto
	Brara.K00318	Peroxidase (PF00141)	Peroxidase	9	287	31.7	6.67	Cyto	Cyto
	Brara.K00699	Peroxidase (PF00141)	Peroxidase	10	327	36.1	8.72	Chlo	Chlo
Chlamydomonas reinhardtii P.A.Dang.	Cre02.g087700	Peroxidase (PF00141)	Peroxidase	10	327	35.6	8.67	Mito/Chlo	Chlo/Mito
	Cre05.g233900	Peroxidase (PF00141)	Peroxidase	8	347	36.4	9.23	Chlo	Chlo/Mito
	Cre06.g285150	Peroxidase (PF00141)	Peroxidase	7	337	35.1	8.95	Chlo/Mito	Chlo/Mito
	Cre09.g401886	Peroxidase (PF00141)	Peroxidase	10	372	39.4	8.63	Chlo	Chlo
Cucumis sativus L.	Cucsa.060660	Peroxidase (PF00141)	Peroxidase	11	413	44.8	7.09	Chlo	Chlo
	Cucsa.162470	Peroxidase (PF00141)	Peroxidase	8	249	27.3	7.74	Chlo/Cyto	Nucl
	Cucsa.213340	Peroxidase (PF00141)	Peroxidase	9	249	27.3	5.43	Cyto	Cyto
	Cucsa.311620	Peroxidase (PF00141)	Peroxidase	11	368	40.2	7.67	Chlo	Chlo
	Cucsa.370590	Peroxidase (PF00141)	Peroxidase	9	286	31.4	6.41	Cyto	Cyto
Eucalyptus grandis W. Hill ex Maiden	Eucgr.A01180	Peroxidase (PF00141)	Peroxidase	9	249	27.4	6.07	Cyto	Cyto
	Eucgr.B02456	Peroxidase (PF00141)	Peroxidase	9	249	27.2	5.29	Cyto	Cyto
	Eucgr.C01740	Peroxidase (PF00141)	Peroxidase	9	369	39.6	8.44	Chlo	Chlo
	Eucgr.F00373	Peroxidase (PF00141)	Peroxidase	11	356	38.3	6.50	Chlo	Chlo
	Eucgr.F04344	Peroxidase (PF00141)	Peroxidase	12	446	48.2	8.71	Chlo	Chlo
	Eucgr.F04344	Peroxidase (PF00141)	Peroxidase	11	397	42.8	8.60	Chlo	Chlo
	Eucgr.I01408	Peroxidase (PF00141)	Peroxidase	9	287	31.5	6.67	Cyto/Chlo	Cyto
Glycine max (L.) Merr.	Glyma.04G248300	Peroxidase (PF00141)	Peroxidase	11	386	41.9	7.06	Chlo	Chlo
	Glyma.06G068200	Peroxidase (PF00141)	Peroxidase	10	319	34.2	7.56	Chlo	Chlo
	Glyma.06G114400	Peroxidase (PF00141)	Peroxidase	12	432	47.0	7.13	Chlo	Chlo

(Continued)

TABLE 5 | Continued

Species name	Phytozome gene ID	Gene/protein features of GPX sequences							
		Protein domain family[a]	Domain family description	Exon no.	Protein length	MW (KDa)	*Theor. pI*	Localization CELLO[b]	Localization WoLF PSORT[b]
	Glyma.11G078400	Peroxidase (PF00141)	Peroxidase	9	280	31.1	9.08	Cyto/Mito	Cyto
	Glyma.12G032300	Peroxidase (PF00141)	Peroxidase	9	287	31.7	6.27	Cyto	Cyto
	Glyma.12G073100	Peroxidase (PF00141)	Peroxidase	9	250	27.1	5.65	Cyto	Cyto
	Glyma.14G177200	Peroxidase (PF00141)	Peroxidase	10	347	37.9	6.76	Extr/Mito/Chlo	Chlo
Gossypium raimondii Ulbr.	Gorai.002G196800	Peroxidase (PF00141)	Peroxidase	9	288	31.7	5.64	Cyto	Cyto
	Gorai.005G254100	Peroxidase (PF00141)	Peroxidase	9	288	31.9	6.67	Cyto	Cyto
	Gorai.009G104500	Peroxidase (PF00141)	Peroxidase	9	250	27.5	5.73	Cyto	Cyto
	Gorai.009G246900	Peroxidase (PF00141)	Peroxidase	11	385	41.7	8.89	Chlo	Chlo
	Gorai.009G420500	Peroxidase (PF00141)	Peroxidase	9	251	27.8	6.01	Cyto	Cyto
	Gorai.010G038200	Peroxidase (PF00141)	Peroxidase	11	355	38.8	7.53	Chlo	Chlo
	Gorai.010G051400	Peroxidase (PF00141)	Peroxidase	12	422	46.0	6.77	Chlo	Chlo
	Gorai.010G115200	Peroxidase (PF00141)	Peroxidase	10	334	36.2	8.17	Chlo	Chlo
Zea mays L.	GRMZM2G004211	Peroxidase (PF00141)	Peroxidase	9	290	32.0	7.72	Cyto/Mito	Cyto
	GRMZM2G006791	Peroxidase (PF00141)	Peroxidase	12	451	48.9	5.60	Chlo	Chlo
	GRMZM2G047968	Peroxidase (PF00141)	Peroxidase	7	223	23.7	9.01	Chlo/Cyto	Mito/Chlo
	GRMZM2G054300	Peroxidase (PF00141)	Peroxidase	9	250	27.3	5.56	Cyto	Cyto
	GRMZM2G120517	Peroxidase (PF00141)	Peroxidase	11	339	37.0	8.86	Mito	Chlo
	GRMZM2G137839	Peroxidase (PF00141)	Peroxidase	9	250	27.3	5.64	Cyto	Cyto
	GRMZM2G156227	Peroxidase (PF00141)	Peroxidase	10	351	38.3	8.62	Mito	Chlo
	GRMZM2G460406	Peroxidase (PF00141)	Peroxidase	8	289	31.6	7.73	Cyto/Chlo	Cyto
Vitis vinifera L.	GSVIVG01008846001	Peroxidase (PF00141)	Peroxidase	11	372	40	7.10	Chlo	Chlo
	GSVIVG01009079001	Peroxidase (PF00141)	Peroxidase	10	344	37.4	6.65	Extr/Chlo	Chlo
	GSVIVG01024035001	Peroxidase (PF00141)	Peroxidase	9	289	31.7	7.72	Chlo/Cyto	Cyto
	GSVIVG01025104001	Peroxidase (PF00141)	Peroxidase	9	250	27.5	5.71	Cyto	Cyto
	GSVIVG01025551001	Peroxidase (PF00141)	Peroxidase	9	253	27.9	5.43	Cyto	Cyto
	GSVIVG01035858001	Peroxidase (PF00141)	Peroxidase	10	330	35.9	6.47	Chlo/Cyto	Chlo
Oryza sativa L.	LOC_Os02g34810	Peroxidase (PF00141)	Peroxidase	12	478	51.1	5.36	Chlo	Chlo
	LOC_Os04g35520	Peroxidase (PF00141)	Peroxidase	11	359	38.3	8.76	Chlo	Chlo
	LOC_Os04g51300	Peroxidase (PF00141)	Peroxidase	11	353	38.1	8.67	Mito/Chlo	Chlo
	LOC_Os07g49400	Peroxidase (PF00141)	Peroxidase	9	251	27.1	5.18	Cyto	Cyto
	LOC_Os08g41090	Peroxidase (PF00141)	Peroxidase	10	331	35.5	6.95	Chlo	Chlo
	LOC_Os08g43560	Peroxidase (PF00141)	Peroxidase	9	291	31.7	7.74	Chlo/Cyto/Mito	Cyto
Medicago truncatula Gaertn.	Medtr3g088160	Peroxidase (PF00141)	Peroxidase	11	436	47.4	9.02	Chlo	Chlo
	Medtr3g088160	Peroxidase (PF00141)	Peroxidase	10	387	42.0	8.73	Chlo	Chlo
	Medtr3g107060	Peroxidase (PF00141)	Peroxidase	10	320	34.7	8.08	Chlo	Mito/Chlo
	Medtr4g061140	Peroxidase (PF00141)	Peroxidase	9	250	27.1	5.52	Cyto	Cyto
	Medtr4g073410	Peroxidase (PF00141)	Peroxidase	9	287	31.7	6.26	Cyto	Chlo/Cyto
	Medtr5g022510	Peroxidase (PF00141)	Peroxidase	9	281	31.4	8.74	Cyto	Cyto
	Medtr5g064610	Peroxidase (PF00141)	Peroxidase	10	353	38.9	8.18	Mito/Nucl	Chlo
Physcomitrella patens (Hedw.) Bruch & Schimp.	Phpat.001G070500	Peroxidase (PF00141)	Peroxidase	11	358	38.4	7.56	Chlo	Chlo
	Phpat.001G104200	Peroxidase (PF00141)	Peroxidase	9	300	32.6	7.01	Chlo	Cyto
	Phpat.001G162800	Peroxidase (PF00141)	Peroxidase	2	440	48.2	8.11	Chlo	Chlo
	Phpat.017G025400	Peroxidase (PF00141)	Peroxidase	11	357	38.4	6.15	Chlo	Chlo
	Phpat.020G011100	Peroxidase (PF00141)	Peroxidase	9	250	27.6	5.66	Cyto	Cyto

(Continued)

TABLE 5 | Continued

Species name	Phytozome gene ID	Gene/protein features of GPX sequences							
		Protein domain family[a]	Domain family description	Exon no.	Protein length	MW (KDa)	*Theor. pI*	Localization CELLO[b]	Localization WoLF PSORT[b]
Phaseolus vulgaris L.	Phvul.008G176700	Peroxidase (PF00141)	Peroxidase	10	347	37.6	6.05	Chlo/Extr	Chlo
	Phvul.009G093000	Peroxidase (PF00141)	Peroxidase	10	317	34.2	8.38	Chlo	Chlo
	Phvul.009G126500	Peroxidase (PF00141)	Peroxidase	12	436	47.8	8.67	Chlo	Chlo
	Phvul.009G126500	Peroxidase (PF00141)	Peroxidase	11	387	42.4	8.51	Chlo	Chlo
	Phvul.011G035000	Peroxidase (PF00141)	Peroxidase	9	287	31.6	7.10	Cyto	Cyto
	Phvul.011G071300	Peroxidase (PF00141)	Peroxidase	9	250	27	5.54	Cyto	Cyto
Populus trichocarpa Torr. & A.Gray ex. Hook.	Potri.004G174500	Peroxidase (PF00141)	Peroxidase	9	286	31.5	6.67	Cyto	Cyto
	Potri.005G161900	Peroxidase (PF00141)	Peroxidase	10	347	37.8	7.59	Chlo/Mito	Chlo
	Potri.005G179200	Peroxidase (PF00141)	Peroxidase	10	345	37.8	5.98	Cyto	Chlo/Mito
	Potri.006G132200	Peroxidase (PF00141)	Peroxidase	9	249	27.4	5.27	Cyto	Cyto
	Potri.006G254500	Peroxidase (PF00141)	Peroxidase	10	337	36.7	8.44	Chlo	Chlo
	Potri.009G015400	Peroxidase (PF00141)	Peroxidase	9	249	27.3	5.53	Cyto	Cyto
	Potri.009G134100	Peroxidase (PF00141)	Peroxidase	9	286	31.4	7.06	Cyto	Cyto
Prunus persica (L.) Batsch	ppa006270m	Peroxidase (PF00141)	Peroxidase	11	420	45.4	8.48	Chlo	Chlo
	ppa008008m	Peroxidase (PF00141)	Peroxidase	10	349	38.4	6.09	Mito/Chlo/Extr	Chlo
	ppa009582m	Peroxidase (PF00141)	Peroxidase	9	286	31.4	6.21	Cyto	Cyto
	ppa010413m	Peroxidase (PF00141)	Peroxidase	9	250	27.3	5.76	Cyto	Cyto
	ppa010426m	Peroxidase (PF00141)	Peroxidase	9	250	27.6	5.37	Cyto	Cyto
	ppa015878m	Peroxidase (PF00141)	Peroxidase	10	319	34.3	6.24	Chlo	Chlo
Sorghum bicolor (L.) Moench	Sobic.001G410200	Peroxidase (PF00141)	Peroxidase	9	250	27.2	5.55	Cyto	Cyto
	Sobic.002G431100	Peroxidase (PF00141)	Peroxidase	9	250	27.1	5.18	Cyto	Cyto
	Sobic.004G175500	Peroxidase (PF00141)	Peroxidase	13	473	51.1	5.03	Chlo	Chlo
	Sobic.006G021100	Peroxidase (PF00141)	Peroxidase	9	476	52.1	8.97	Nucl	Chlo
	Sobic.006G084400	Peroxidase (PF00141)	Peroxidase	11	344	37.2	8.60	Mito/Chlo	Chlo
	Sobic.006G204000	Peroxidase (PF00141)	Peroxidase	11	395	42.9	8.74	Mito/Chlo	Chlo
	Sobic.007G177000	Peroxidase (PF00141)	Peroxidase	8	289	31.5	7.73	Cyto	Cyto
	Sobic.007G205600	Peroxidase (PF00141)	Peroxidase	10	333	36.2	7.58	Chlo	Chlo
Solanum lycopersicum L.	Solyc01g111510	Peroxidase (PF00141)	Peroxidase	8	287	31.6	7.10	Cyto	Cyto
	Solyc04g074640	Peroxidase (PF00141)	Peroxidase	10	345	37.6	7.60	Chlo/Mito	Chlo
	Solyc06g005150	Peroxidase (PF00141)	Peroxidase	9	250	27.3	5.86	Cyto	Cyto
	Solyc06g060260	Peroxidase (PF00141)	Peroxidase	10	345	37.8	8.48	Chlo	Chlo
	Solyc08g059760	Peroxidase (PF00141)	Peroxidase	10	326	35.4	5.65	Chlo	Chlo
	Solyc09g007270	Peroxidase (PF00141)	Peroxidase	9	250	27.6	5.63	Cyto	Cyto
	Solyc11g018550	Peroxidase (PF00141)	Peroxidase	12	421	46.0	8.20	Chlo	Chlo

[a] *Protein domain families were searched in Pfam database.*

[b] *Cyto, Cytosolic; Chlo, Chloroplastic; Mito, Mitochondrial; Nucl, Nuclear; Extr, Extracellular.*

More than one localization specified in a single column also shows the significance of other entries in order.

thereby indicated the presence of transit residues (**Table 5**). Molecular cloning studies from *A. thaliana* have demonstrated that APX1, APX2, and APX6 are polypeptides of 250, 251, and 329 amino acids, respectively, with cytosolic localizations but without transit peptide (Davletova et al., 2005; Jones et al., 2009; Aryal et al., 2011). APX3 and APX5 consisted of 287 and 279 amino acids, respectively, with peroxisomal localizations; however, sites of transit peptide residues are not precisely specified (Panchuk et al., 2002; Narendra et al., 2006; Bienvenut et al., 2012). APX4 is a 349 amino acids protein with chloroplastic localization, including 1–82 residues as transit peptide from the N-terminal site (Kieselbach et al., 2000; Panchuk et al., 2005; Aryal et al., 2011). APXT is a 426 amino acids chloroplastic protein, including 1–78 residues of transit peptide (Theologis et al., 2000; Panchuk et al., 2005). APXS consists of 372 amino acids with chloroplastic and/or mitochondrial localizations, but

the exact site of the transit peptide is not specified (Jespersen et al., 1997; Mayer et al., 1999; Chew et al., 2003). In the present study, multiple-alignment of APX sequences revealed that chloroplastic/mitochondrial-related APXs contained the transit peptide residues in approximately 1–90 amino acids from the N-terminal site while cytosolic APXs did not have any putative transit peptide (Supplementary Figure S4). Thus, the analyzed APX sequences were gathered in two main groups based on subcellular localizations, such as chloroplastic/mitochondrial APXs (i) and cytosolic APXs (ii).

In addition, the regions corresponding to the transit peptide sites in analyzed sequences did not demonstrate any particular pattern. This could indicate that less conservancy in transit peptides may be associated with the functional diversities of APXs at various targets. Besides, APX transcripts mainly consisted of 8–12 exons, supporting the relatively less conserved structure of APXs compared to GPXs. However, alignment analysis also demonstrated the presence of a considerable degree of conserved residues in the main sites of enzyme (Supplementary Figure S5). Moreover, to analyze the availability of any conserved motif pattern/s in APX sequences, the most conserved five motif sequences of APX homologs were searched using MEME tool (**Table 6**). Motif 1 was 29 residues long, motif 2 and 4 were 21 residues, motif 3 was 32 residues, and motif 5 was 25 residues in length. However, only motifs 2 and 3 had a relation with the peroxidase (PF00141) protein family, and in this case were present in most of the sequences. This could indicate the highly conserved structures of APX sequences at those sites with peroxidase activity.

Furthermore, alignment analysis also demonstrated that Asp (D) and Gly-Gly (GG) residues are strictly conserved in all aligned sequences, indicating their potential functions in enzyme activity and/or stability (Supplementary Figure S6). To infer a functional relationship between these conserved residues and APX sequences, we searched for the known binding residues of model organism *Arabidopsis* APXs in the UniProtKB database (http://www.uniprot.org/uniprot/). The following residues were reported as potential active and metal binding residues for *Arabidopsis* GPX1-6, APXT, and APXS: APX1 (Arg-38, His-42, His-163, Thr-164, Thr-180, Asn-182, Ile-185, Asp-187), APX2 (Arg-39, His-43, His-163, Thr-164, Thr-180, Asn-182, Ile-185, Asp-187), APX3 (Arg-36, His-40, His-160, Thr-161, Thr-177, Asp-184), APX5 (Arg-35, His-39, His-158, Thr-159, Thr-175, Asp-182), APX6 (Arg-119, His-123, His-224), APXT (Arg-108, His-112, His-241, Thr-242, Thr-274, Asp-281), and APXS (Arg-129, His-133, His-262, Thr-263, Thr-295, Asp-302). These active and metal binding residues did not correspond to any of the strictly conserved residues in analyzed APX sequences but they were found to be conserved at considerable rates. However, when taken into consideration that some of the strictly conserved residues in analyzed GPX sequences correspond to the catalytic sites of the enzymes, we can make an inference that these strictly conserved residues in APX sequences may also be associated with the peroxidase activity of the enzyme.

Phylogenetic Analysis of APXs

To analyze the evolutionary relationship between identified APX homologs, the phylogenetic tree was constructed by MEGA 6 using the Maximum Likelihood (ML) method with 1000 bootstraps (**Figure 5**). The constructed tree was divided into five major groups based on the tree topology, and each group was indicated with a different color segment. Blue, red, and green segments included the chloroplast/mitochondria-related APXs with relatively longer, medium and short sequences, respectively, whereas cyan and yellow segments mainly contained longer and shorter cytosolic APX sequences, respectively. Annotation of each segment was based on the consensus of two subcellular localization servers, CELLO and WoLF PSORT, as well as tree topology for ambiguous sequences. Overall, it was observed that cytosolic-related APXs clustered together, while alternatively chloroplast/mitochondrial-related APXs were together. In addition, in clustering of sequences at sub-branches was primarily based on the sequence length and monocot/dicot separation. However, there were considerable variations between sequences, even those belonging to the same subcellular localization. It is thought that these sequence variations could be attributed to the various functional diversities of APXs and/or be associated with different subcellular localizations. Moreover, some sequences were also available with different subcellular localizations in the same clade, indicating the possibility of gene duplication events in formation of some *APX* genes. The gene duplication events were searched based on the previously designated protocol (Gu et al., 2002). In doing so, several segmental and tandem duplications were identified between some APX pairs (**Table 7**). The identified segmental or tandem duplications in *APX* genes were observed between either chloroplastic and chloroplastic, or cytosolic and cytosolic forms. This could indicate the possibility of gene duplication events in the formation of close *APX* homologs.

TABLE 6 | Most conserved five motifs of ascorbate peroxidase (APX) homologs in 18 plant species.

Motif	Width	Identified site no.	Sequence	Protein domain family[a]
1	29	120 of 120	CHPIMLRLAWHDAGTYDKNTKTWGPNGSI	Not found
2	21	101 of 120	MGLNDQDIVALSGGHTLGRCH	Peroxidase (PF00141)
3	32	119 of 120	IITYADLYQLAGVVAVEVCGGPTIPMHCGRND	Peroxidase (PF00141)
4	21	118 of 120	DPEFRPWVEKYAEDQDAFFRD	Not found
5	25	84 of 120	ERSGFEQPWTVNWLKFDNSYFKEIL	Not found

[a] *Protein domain families have been searched in Pfam database.*

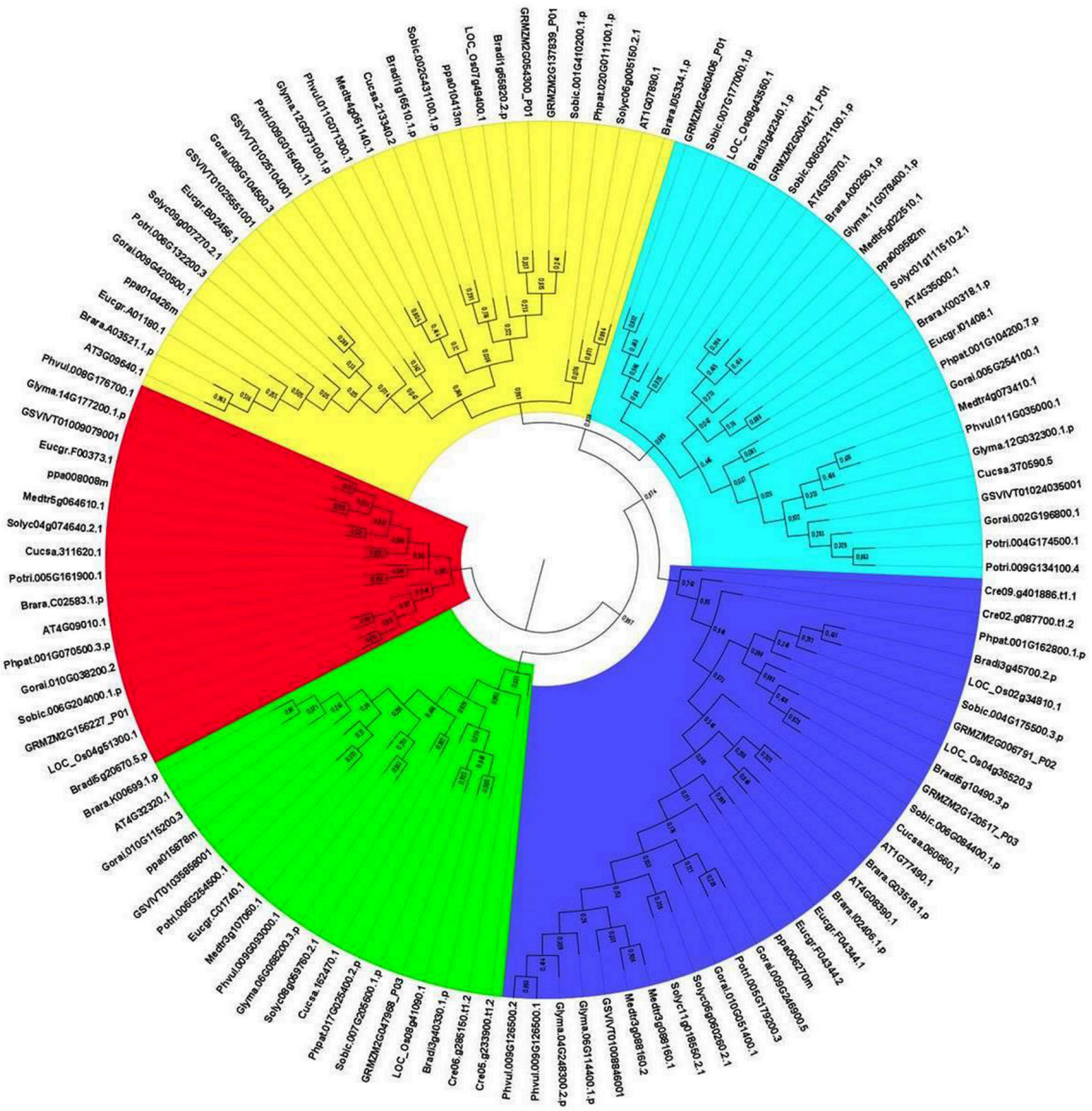

FIGURE 5 | Phylogenetic tree of ascorbate peroxidase (APX) homologs from 18 plant species. Tree was constructed by MEGA 6 using Maximum likelihood (ML) method with 1000 bootstraps. Segment classification based on the consensus of two subcellular localization servers, CELLO and WoLF PSORT as well as tree topology for ambiguous sequences. Blue, red, and green segments include chloroplast/mitochondrial related APXs with mainly longer, medium and short sequences, respectively, while cyan and yellow segments contain longer and shorter cytosolic APX sequences, respectively.

Expression Profile Analysis of APXs

The gene expression profiles of *APX*s were analyzed at 105 anatomical parts and 10 developmental stage levels using model organism *A. thaliana APX*s from Genevestigator platform (**Figure 6**). Eight *Arabidopsis* genes, namely APX1 (AT1G07890), APX2 (AT3G09640), APX3 (AT4G35000), APX4 (AT4G09010), APX5 (AT4G35970), APX6 (AT4G32320), TAPX (AT1G77490), and SAPX (AT4G08390), were retrieved from the "Affymetrix Arabidopsis ATH1 Genome Array" platform using the Genevestigator interface. Thereafter, conditions and genes with similar profiles were comparatively analyzed using Hierarchical clustering tool with Euclidean distance method.

At the anatomical level (**Figure 6A**), *APX* genes were expressed in almost all analyzed tissues of *Arabidopsis* with various folds. It was clear that the expression levels of genes were closely related with the expressed tissue type/s. For example, both cytosolic *APX1* and chloroplastic/mitochondrial *SAPX* had significantly higher expression in actively growing zones, as well as many root and root protoplast-related structures. *APX3, APX4, APX6,* and *TAPX* were expressed in various shoot, bud, leaf, flower and seed related tissues at considerable rates. All these indicated that stress factors, actively growing tissues as well as normal physiological and metabolic changes could induce the expression of *APX* genes in tissue-dependent way. All these

TABLE 7 | The segmental and tandem duplications in some ascorbate peroxidase (APX) pairs.

Duplication type	Species name	Duplicated pairs
Segmental duplication Pairs	*Brachypodium distachyon* (L.) P.Beauv.	Bradi5g10490-Bradi3g45700
	Eucalyptus grandis W. Hill ex Maiden	Eucgr.A01180-Eucgr.B02456
	Glycine max (L.) Merr.	Glyma.06G114400-Glyma.04G248300
		Glyma.11G078400-Glyma.12G032300
	Gossypium raimondii Ulbr.	Gorai.002G196800-Gorai.005G254100
		Gorai.009G246900-Gorai.010G051400
	Vitis vinifera L.	GSVIVG01025104001-GSVIVG01025551001
	Oryza sativa L.	LOC_Os04g35520-LOC_Os02g34810
	Populus trichocarpa Torr. & A.Gray ex. Hook.	Potri.004G174500-Potri.009G134100
		Potri.006G132200-Potri.009G015400
	Prunus persica (L.) Batsch	ppa010431m.g-ppa010426m.g
	Sorghum bicolor (L.) Moench	Sobic.001G410200-Sobic.002G431100
		Sobic.006G084400-Sobic.004G175500
		Sobic.007G177000-Sobic.006G021100,
	Solanum lycopersicum L.	Solyc06g005150.2-Solyc09g007270.2
		Solyc06g060260.2-Solyc11g018550.2
Tandem duplication Pairs	*Brachypodium distachyon (L.) P.Beauv.*	Bradi1g16510-Bradi1g65820
	Gossypium raimondii Ulbr.	Gorai.009G104500-Gorai.009G420500
	Zea mays L.	GRMZM2G006791-GRMZM2G120517
		GRMZM2G054300-GRMZM2G137839

metabolic activities or their related consequences could exert the stresses to the cells. Many studies have further demonstrated that abiotic/abiotic stress factors such as heavy metal, drought, water, heat, cellular H_2O_2 level, oxidative state of the cell could increase the expression of *APX* genes to either suppress or eliminate the stressors (Ishikawa and Shigeoka, 2008; Koussevitzky et al., 2008; Yang et al., 2008; Petrov and Van Breusegem, 2012). For example, overexpression of *Solanum melongena APX6* in transgenic *O. sativa* seedlings demonstrated high flood tolerance, reduced oxidative injury and fast plant growth rates (Chiang et al., 2015a). APX regulation by nitric oxide (NO) as a redox indicator in oxidative stress or as part of hormone induced signaling pathway in lateral root development were demonstrated (Correa-Aragunde et al., 2015). *S*-nitrosylation of *Arabidopsis APX1* at Cys32 increased the H_2O_2 scavenging activity of enzyme, resulting in improved oxidative stress tolerance (Yang et al., 2015). Overexpression of *APX* and *Cu/Zn SOD* increased the drought resistance and recovery capacity from drought stress in *Ipomoea batatas* (Lu et al., 2015). Overexpressed *Brassica campestris* APX gene in transgenic *Arabidopsis* enhanced the heat tolerance *via* elimination of H_2O_2 (Chiang et al., 2015b). Therefore, increased APX activity in cells is an indicator of the presence of stress factors. At the developmental level (**Figure 6B**), the expression profile of *Arabidopsis APXs* was analyzed at 10 developmental stages: senescence, mature siliques, flowers and siliques, developed flower, young rosette, germinated seed, seedling, bolting, young flower, and developed rosette. In all developmental stages, *APXs* were relatively expressed. However, the expression pattern in senescence was slightly different from other developmental stages, notably cytosolic *APX6* showed the highest expression. Interestingly, the *Arabidopsis GPX6* gene also demonstrated the highest expression fold at senescence stage, inferring the possibility of functional similarities of these two enzymes. Overall, the expression profile and fold of *APX*s in various tissues and stages show that cells are constantly put under stress even with normal physiological and metabolic changes, requiring plants to eliminate these stressors.

3D Structure Analysis of APXs

3D models of eight identified *Arabidopsis* APX sequences were constructed by using Phyre2 server (**Figure 7**). The modeled sequences were AT1G07890.1 (*APX1*), AT3G09640.1 (*APX2*), AT4G35000.1 (*APX3*), AT4G09010.1 (*APX4*), AT4G35970.1 (*APX5*), AT4G32320.1 (*APX6*), AT1G77490.1 (*APXT*), and AT4G08390.1 (*APXS*). In modeling, six templates such as 1APX:A (*APX1*), 1OAF:A (*APX2*, *APX3* and *APX5*), 3RRW:B (*APX4*), 1BGP:A (*APX6*), 1ITK:B (*APXT*), and 1IYN:A (*APXS*) were used to maximize the alignment coverage, percentage identity, and confidence for the submitted sequences. Predicted models showed the ≥96% of residues were within the allowed region in Ramachandran plot, indicating that structures were acceptably high in quality. To analyze the divergence or similarity in generated models, the structures were superposed in order to calculate the percentage of structural overlap and RMSD values (**Table 8**). The superposition of APX sequences demonstrated that APX2-APX3, APX2-APX5, and APX3-APX5 pairs have highly conserved structural overlap (100%) with 0.00, 0.38, and 0.38 RMSD values, respectively. These conserved pairs primarily shared the cytosolic and/or peroxisomal localizations, inferring the possibility of a functional relationship between them. In addition, the APX1-APX2, 3, and 5 pairs had very high structural similarity with ≥99 structural overlaps. Therefore, it could be deduced that APX members topologically demonstrated highly conserved structures, despite their functional diversities in different cellular compartments.

Interaction Partner Analysis of APXs

The interactome network was constructed for 10 putative interactors of *Arabidopsis* cytosolic APX1 using Cytoscape with STRING data (**Figure 8**). MDHAR (monodehydroascorbate reductase), GPX2 (glutathione peroxidase 2), DHAR1 (dehydroascorbate reductase), MDAR1 (monodehydroascorbate reductase 1), RHL41 (zinc finger protein ZAT12), ATPQ (ATP synthase subunit d), FBP (fructose-1,6-bisphosphatase), ATMDAR2 [monodehydroascorbate reductase (NADH)],

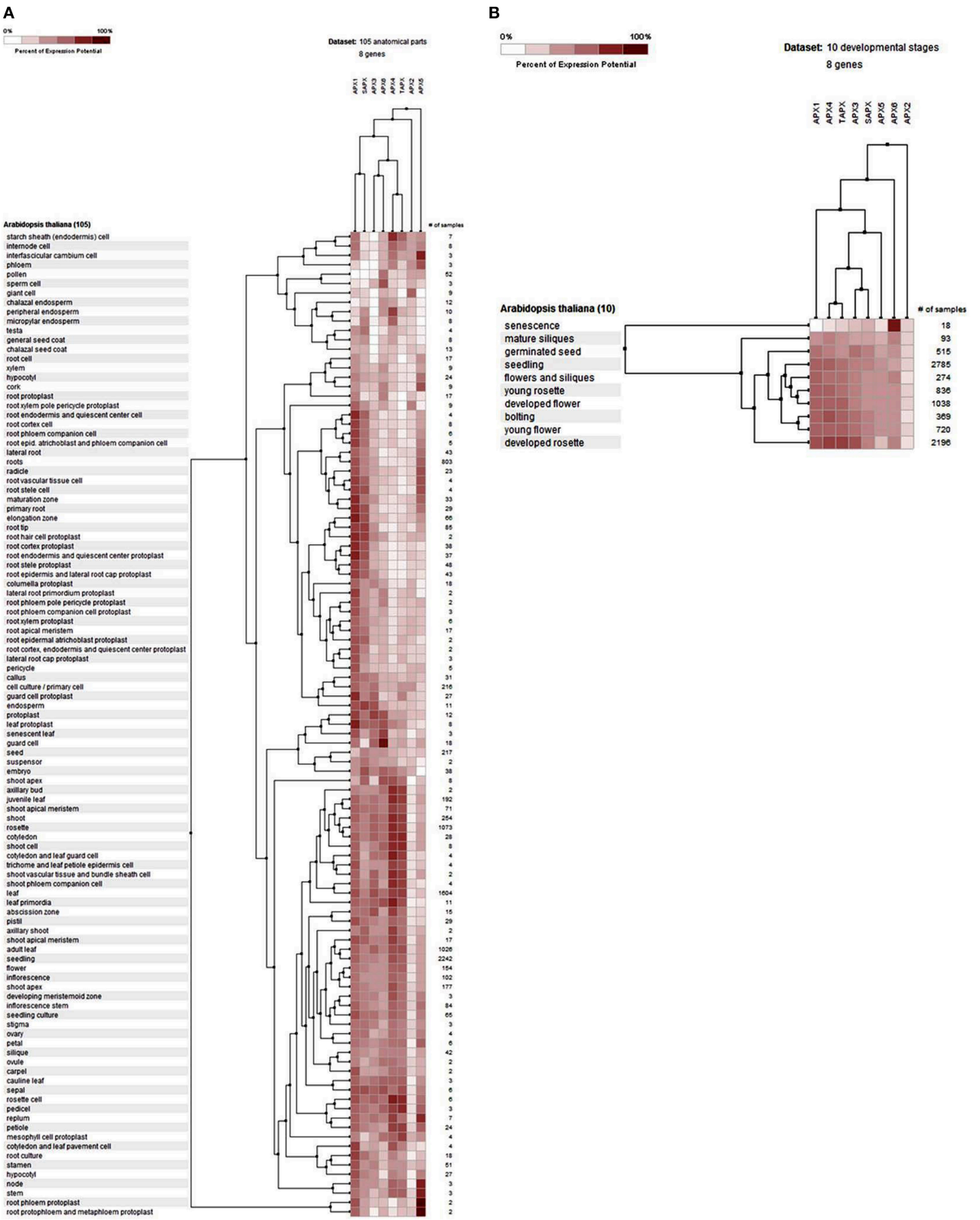

FIGURE 6 | Expression profile of *Arabidopsis* ascorbate peroxidase *APX1-6, TAPX* and *SAPX* genes at 105 anatomical parts (A) and 10 developmental stage levels (B). Genes and conditions with similar profiles were comparatively analyzed using hierarchical clustering tool with Euclidean distance method at Genevestigator platform.

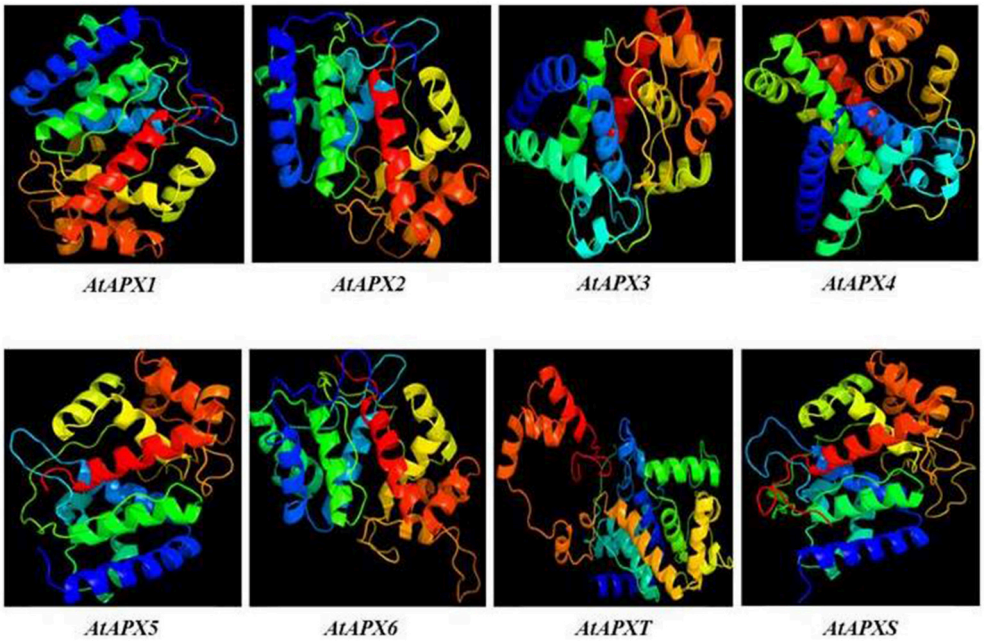

FIGURE 7 | 3D models of predicted *Arabidopsis* ascorbate peroxidase APX1-6, APXT, and APXS sequences. Models were constructed by using Phyre[2] server for AT1G07890.1 (APX1), AT3G09640.1 (APX2), AT4G35000.1 (APX3), AT4G09010.1 (APX4), AT4G35970.1 (APX5), AT4G32320.1 (APX6), AT1G77490.1 (APXT), and AT4G08390.1 (APXS) sequences, and colored by rainbow from N- to C-terminus.

TABLE 8 | Structural overlap (%)/RMSD values in superposed *Arabidopsis* ascorbate peroxidases (APXs).

	APX1	APX2	APX3	APX4	APX5	APX6	APXS	APXT
APX1	–	99.19/0.43	99.59/0.41	75.10/1.75	99.58/0.51	81.53/1.52	95.18/0.95	89.16/1.49
APX2	99.19/0.41	–	100.00/0.00	75.0071.78	100.00/0.38	81.45/1.58	95.16/0.86	87.90/1.35
APX3	99.59/0.41	100.00/0.00	–	75.31/1.85	100.00/0.38	82.30/1.56	97.12/0.86	88.48/1.38
APX4	75.10/1.75	75.40/1.73	73.66/1.91	–	73.22/1.92	72.29/1.88	75.79/1.72	66.27/1.83
APX5	99.58/0.48	100.00/0.38	100.00/0.38	74.48/1.85	–	81.17/1.67	97.49/0.90	89.12/1.31
APX6	82.73/1.57	82.26/1.70	83.13/1.68	69.88/1.82	84.10/1.70	–	81.12/1.50	73.90/1.66
APXS	95.18/1.00	95.97/0.97	97.12/1.00	75.00/1.73	97.49/1.05	82.73/1.55	–	83.52/1.38
APXT	87.55/1.47	89.52/1.36	89.71/1.32	67.46/1.87	90.79/1.32	74.70/1.80	82.42/1.34	–

Red-color highlighted pairs show the highly conserved structural overlaps.

CYTC-1 (cytochrome c-1), and CYTC-2 (cytochrome c-2) proteins were predicted as the main interaction partners of *Arabidopsis* cytosolic APX1. MDHAR, MDAR1 and ATMDAR2 catalyze the conversion of monodehydroascorbate to ascorbate (Chew et al., 2003). GPX2 is a type of H_2O_2-scavenging enzyme and a crucial component in reactive oxygen network (Tanaka et al., 2005). DHAR1 has dual functions: soluble protein, it demonstrates GSH-dependent thiol transferase and dehydroascorbate (DHA) reductase activities, and is involved in redox homeostasis. As a peripheral membrane protein, it functions as voltage-gated ion channel (Dixon et al., 2002; Sasaki-Sekimoto et al., 2005). RHL41 affects in modulation of light acclimation, and cold and oxidative stress responses (Rizhsky et al., 2004; Davletova et al., 2005). ATPQ functions in ATP production (Carraro et al., 2014). FBP is reported to be a key component in photosynthetic sucrose synthesis (Cho et al., 2012). CYTC-1 and CYTC-2 are electron carrier proteins related with mitochondrial electron transport chain (Welchen and Gonzalez, 2005). In light of putative interaction partner analysis, it was apparent that *Arabidopsis* cytosolic APX1 is either directly or indirectly associated with redox homeostasis, stress adaptation and photosynthesis/respiration-related pathways. This could also help in better understanding the functional role of APX1 in various plant defense mechanisms.

Comparison of APX and GPX Sequences

A strict homology search of *Arabidopsis* GPX1-8 sequences in proteome datasets of 18 specified plant species has given a total of 87 putative GPX sequences; however, homology search of *Arabidopsis* APX1-6, APXT, and APXS in proteome

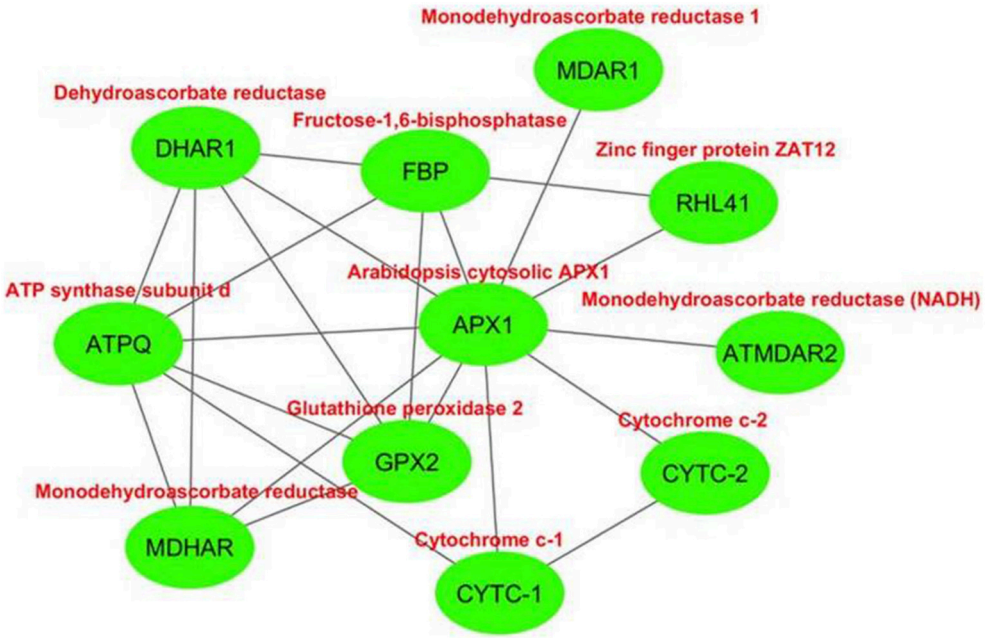

FIGURE 8 | Predicted 10 interaction partners of *Arabidopsis* cytosolic ascorbate peroxidase APX1. Interactome was generated using cytoscape with STRING data. MDHAR (monodehydroascorbate reductase), GPX2 (glutathione peroxidase 2), DHAR1 (dehydroascorbate reductase), MDAR1 (monodehydroascorbate reductase 1), RHL41 (zinc finger protein ZAT12), ATPQ (ATP synthase subunit d), FBP (fructose-1,6-bisphosphatase), ATMDAR2 (monodehydroascorbate reductase (NADH)), CYTC-1 (cytochrome c-1), and CYTC-2 (cytochrome c-2) proteins were predicted as main interaction partners of *Arabidopsis* cytosolic APX1.

datasets of these species identified a total of 120 putative APXs (**Tables 1**, **5**). Sequences of GPX homologs contained the GPX (PF00255) protein family domain while APX homologs included the peroxidase (PF00141) domain. *GPX* genes encoded a protein of 166–262 residues with 18.4–29.7 kDa molecular weight and 4.59–9.60 *pI* value, while *APX*s encoded a polypeptide of 197–478 residues with 23.7–52.1 kDa molecular weight and 5.03–9.23 *pI* value. GPX transcripts mainly contained six exons; whereas, APX usually had 8–12 exons, implicating the relatively less conserved structure of APXs compared to GPXs. Sequence variations in GPX and APX homologs primarily derived from the "transit peptide" residues between organelle and non-organelle related sequences. Besides, regions corresponding to transit peptide sites in APX/GPX sequences did not demonstrate any particular pattern, indicating the less conserved structure of transit peptides thereby the functional diversities of APXs/GPXs at various targets. In addition, multiple-alignment analyses demonstrated the presence of a considerable degree of conserved residues in main sites of both enzymes. In GPX phylogeny, cytosolic-, nuclear-, extra cellular,- and plasma membrane-related GPXs were relatedly clustered while chloroplast/mitochondrial-related GPXs grouped together. APX phylogeny also showed similar clustering pattern, in which cytosolic-related APXs were relatedly clustered while chloroplast/mitochondrial-related APXs were together. This indicates that presence/absence of "transit peptide" residues was the main determinant in phylogenetic distribution of APX/GPX sequences. Moreover, presence of sequences with different subcellular localizations in the same phylogenetic group inferred the possibility of gene duplication events in formation of some APX/GPX sequences. Several segmental duplications were identified in some GPX pairs, while several segmental and tandem duplications were available in some APX pairs. Expression profiles of *GPX* and *APX* genes in model organism *Arabidopsis* indicated that stress factors, actively growing tissues, even normal physiological, and metabolic changes could induce the expression of *APX/GPX* genes. Interactome analyses of *Arabidopsis* cytosolic APX1 and GPX2 also implicated that both enzymes are closely related with antioxidant and redox homeostasis, secondary metabolite metabolisms and stress adaptation thereby supporting the functional roles of APXs/GPXs in H_2O_2-scavenging and plant defense. Despite of some minor variations, APX and GPX members, they topologically demonstrated highly conserved structure.

CONCLUSIONS

The presence or absence of transit peptide residues are the main contributing factors in subcellular localization and phylogenetic distribution of APX/GPXs. The APX/GPX expression is highly associated with the metabolic state of the cells. In addition, there are grounds for belief that these two enzymes work together in various pathways such as antioxidant and secondary metabolite metabolisms, redox homeostasis, stress adaptation,

and photosynthesis/respiration. This also supports the functional role of these enzymes in H_2O_2-scavenging, thereby implicating their importance in the plant defense. However, further molecular and physiological studies are required to elucidate the various functional roles of APX/GPX isoforms.

AUTHOR CONTRIBUTIONS

IK and EF contributed to the study conception and design. KK performed experiments and collected data. Data analysis and interpretation were performed by RV. IK, EF, KK, and RV prepared, and NA performed critical reading and revision of the manuscript. IO and EF supervised and MO coordinated this work. All the authors read and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00301

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Hydrogen Peroxide, Signaling in Disguise during Metal Phytotoxicity

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Plants exposed to excess metals are challenged by an increased generation of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$). The mechanisms underlying this oxidative challenge are often dependent on metal-specific properties and might play a role in stress perception, signaling and acclimation. Although ROS were initially considered as toxic compounds causing damage to various cellular structures, their role as signaling molecules became a topic of intense research over the last decade. Hydrogen peroxide in particular is important in signaling because of its relatively low toxicity, long lifespan and its ability to cross cellular membranes. The delicate balance between its production and scavenging by a plethora of enzymatic and metabolic antioxidants is crucial in the onset of diverse signaling cascades that finally lead to plant acclimation to metal stress. In this review, our current knowledge on the dual role of ROS in metal-exposed plants is presented. Evidence for a relationship between H_2O_2 and plant metal tolerance is provided. Furthermore, emphasis is put on recent advances in understanding cellular damage and downstream signaling responses as a result of metal-induced H_2O_2 production. Finally, special attention is paid to the interaction between H_2O_2 and other signaling components such as transcription factors, mitogen-activated protein kinases, phytohormones and regulating systems (e.g. microRNAs). These responses potentially underlie metal-induced senescence in plants. Elucidating the signaling network activated during metal stress is a pivotal step to make progress in applied technologies like phytoremediation of polluted soils.

Keywords: metals, hydrogen peroxide, oxidative stress, damage, signaling, crosstalk

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THE RELATIONSHIP BETWEEN METALS AND OXIDATIVE STRESS IN PLANTS

Pollution of soils, air, (ground)water and sediments with toxic metals is one of the major problems our industrialized world is currently facing. Naturally occurring levels of these metals have been significantly exceeded by anthropogenic activities over the past two centuries. Mining and industry, as well as the use of phosphate fertilizers and sewage sludge in agriculture have jointly contributed to an increased production and emission of metals. As opposed to many organic contaminants, metals are non-biodegradable, resulting in their extended persistence in the environment. In addition, food and feed crop plants facilitate the entry of toxic metals into the food chain, thereby leading to bio-enrichment and enhanced risks for human health (Cuypers et al., 2009;

Sharma and Dietz, 2009). The latter has been demonstrated by a plethora of *in vitro*, *in vivo* and epidemiological studies, revealing that the highest health risks are associated with exposure to cadmium (Cd), lead (Pb) and mercury (Hg). Adverse metal-induced health effects are wide-ranging, for example with kidney damage, bone effects and cancer related to human Cd exposure (Järup, 2003; Nair et al., 2013). Nevertheless, metal exposure persists and even increases in less developed countries (Järup, 2003), urging the need to remediate metal-polluted soils.

Metals are categorized as essential or non-essential for plant growth, with different dose-response curves for both classes (Lin and Aarts, 2012). Essential micronutrients such as copper (Cu), iron (Fe), nickel (Ni) and zinc (Zn) function as cofactors in over 1500 proteins crucial for the plant's metabolism. For example, Cu is cardinal for photosynthesis and mitochondrial respiration, while Zn-containing enzymes are important regulators of transcription and translation. For that reason, either too low or high levels of these essential metals would adversely affect plant growth and development (Hänsch and Mendel, 2009; Pilon et al., 2009). To avoid both deficiency and excess, plant cells possess different mechanisms to tightly control the concentrations of essential metals (Lin and Aarts, 2012). However, even low concentrations of non-essential metals such as Cd, Pb and Hg disturb biochemical and physiological processes and decrease plant productivity (Lin and Aarts, 2012).

Sharma and Dietz (2009) have described three major mechanisms underlying metal toxicity in plants. First, different metals show a high affinity toward sulfur or nitrogen donors within proteins, which might interfere with cellular metabolism. Metals are also able to displace essential cations from their specific binding sites within an enzyme. For example, Cd^{2+} was suggested to competitively bind to the essential calcium (Ca^{2+}) site in photosystem II during photoactivation (Faller et al., 2005). Finally, multiple studies have demonstrated that exposure of plants to a diverse array of metals elicits oxidative stress, indicating a misbalance between the production and neutralization of reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$) (Schützendübel and Polle, 2002; Sharma and Dietz, 2009). In view of the different chemical properties of metals, two modes of action can be distinguished. Under physiological conditions, redox-active metals such as Cu and Fe exist in different oxidation states ($Cu^{+/2+}$ and $Fe^{2+/3+}$). This enables both metals to directly participate in the Fenton and Haber-Weiss reactions, finally leading to the formation of highly toxic $^{\bullet}OH$ radicals from H_2O_2 (**Figure 1**; Schützendübel and Polle, 2002; Hänsch and Mendel, 2009; Sharma and Dietz, 2009). On the other hand, physiologically non-redox-active metals such as Cd, Hg, and Zn only indirectly contribute to increased ROS production, for example by depleting or inhibiting cellular antioxidants (**Figure 1**; Schützendübel and Polle, 2002; Sharma and Dietz, 2009).

The term "oxidative stress" implies a harmful process, which is mainly related to the oxidizing nature of ROS. However, intense research over the past decades has shifted this paradigm, pointing toward a dual role for ROS as damaging vs. signaling compounds (Foyer and Noctor, 2005). Currently, ROS and H_2O_2 in particular are considered as essential components of signal transduction used by plants to respond to developmental and environmental cues. In this review, it is our intent to provide an overview of the experimental evidence underlying a dual role for H_2O_2 during metal stress in plants. Within this framework, both H_2O_2-induced damage and signaling—including its targets and interaction with other signaling pathways and regulating systems—are highlighted. Ultimately, the term "oxidative challenge" is preferred, as this implies the harmful vs. beneficial effects of H_2O_2 produced in metal-exposed plants (**Figure 2**).

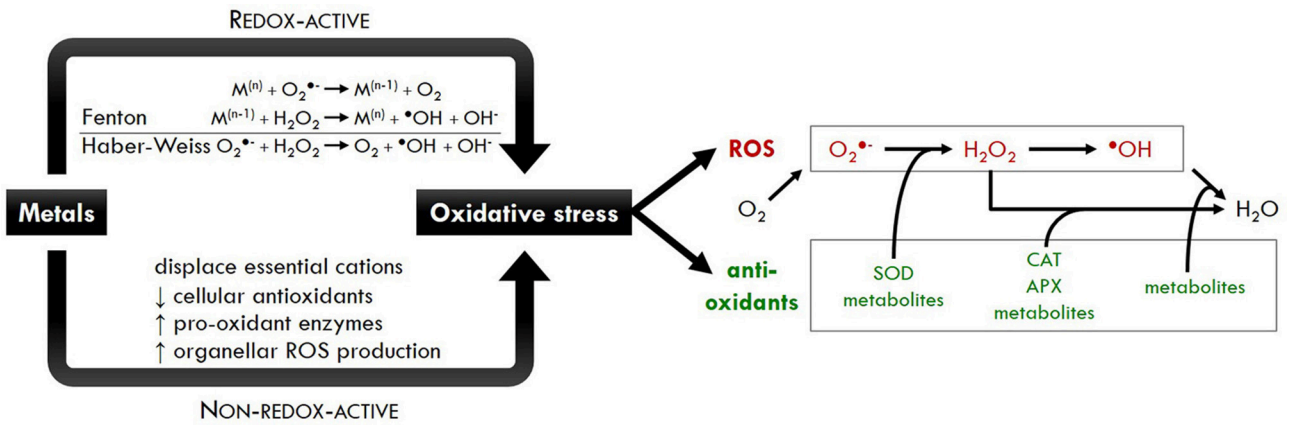

FIGURE 1 | Schematic overview of metal-induced oxidative stress. Redox-active metals such as Cu and Fe can participate in the Fenton and Haber-Weiss reactions, finally leading to the formation of highly toxic $^{\bullet}OH$ radicals from H_2O_2. On the other hand, non-redox-active metals such as Cd and Zn can only indirectly contribute to ROS production by (1) displacing essential cations, (2) depleting cellular antioxidants, (3) increasing the activity of ROS producing enzymes and/or (4) enhancing ROS production in organelles. The net result for both classes of metals is the induction of oxidative stress, an imbalance between ROS and antioxidants in favor of the former. Abbreviations: APX, ascorbate peroxidase; CAT, catalase; H_2O_2, hydrogen peroxide; $M^{(n)}$, oxidized redox-active metal; $M^{(n-1)}$, reduced redox-active metal; $O_2^{\bullet -}$, superoxide; OH^-, hydroxide ion; $^{\bullet}OH$, hydroxyl radical; SOD, superoxide dismutase.

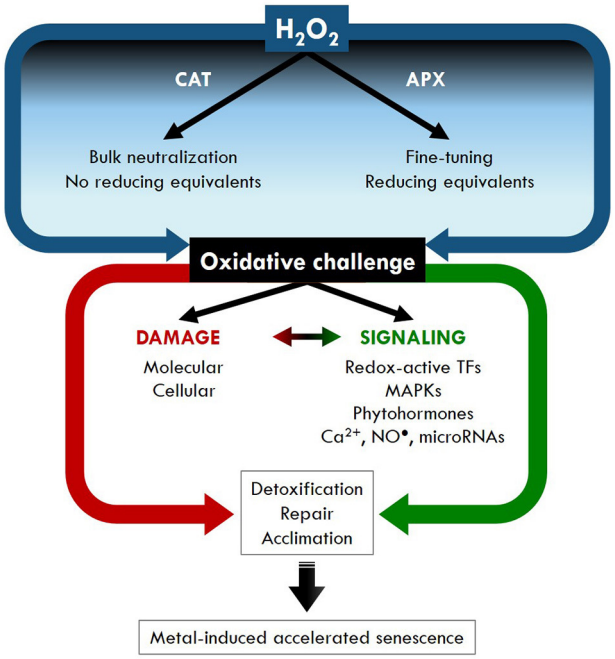

FIGURE 2 | Generalized model for the central role of hydrogen peroxide (H_2O_2) in metal-induced oxidative damage vs. signaling responses. Ascorbate peroxidase (APX) and catalase (CAT) are the two most important enzymes that counterbalance metal-induced H_2O_2 production in plants. However, APX has a much higher affinity for H_2O_2 as compared to CAT. Therefore, the former enzyme is considered to be primarily involved in the fine-tuning of H_2O_2 levels crucial for their signaling function. Conversely, CAT is important for the bulk removal of excess H_2O_2 produced in stressed plants. In addition, while APX uses AsA as a reducing agent to detoxify H_2O_2, CAT does not need any reducing equivalents. Depending on its extent, the metal-induced rise in H_2O_2 content can lead to molecular and cellular damage and/or signaling. Different studies highlight the interaction between ROS/H_2O_2 and signaling components such as redox-active transcription factors (TFs), mitogen-activated protein kinases (MAPKs), phytohormones, Ca^{2+}, $NO^{\bullet}$ and regulating systems like miRNAs. Finally, detoxification, repair and acclimation responses are activated, with accelerated senescence as a potential last resort in metal-exposed plants.

HYDROGEN PEROXIDE, A SIGNALING MOLECULE IN DISGUISE

Both energy transfer to as well as incomplete reduction of O_2 generate ROS such as singlet oxygen ($^1O_2$) and $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ respectively. These reactive intermediates are byproducts of physiological processes such as photosynthesis and respiration, with a high oxidizing potential toward DNA, lipids and proteins. However, not all ROS are equally reactive, with $O_2^{\bullet-}$ and H_2O_2 being rather selective in their reactions and $^{\bullet}OH$ attacking all molecules in its surroundings (Halliwell, 2006; Møller et al., 2007). Under steady-state conditions, antioxidant enzymes and metabolites tightly control ROS concentrations in different cellular compartments to prevent oxidative damage (Mittler et al., 2004, 2011). In addition, plants have developed a way to employ low levels of ROS as signaling compounds to appropriately and coordinately respond to developmental as well as environmental cues (Petrov and Van Breusegem, 2012). It has long been known that different biotic (e.g. pathogen attack) and abiotic (e.g. drought, salinity, heat and metal stress) stimuli increase ROS generation in plants, leading to a misbalance between ROS and antioxidants in favor of the former (Dat et al., 2000; Apel and Hirt, 2004). Especially under these conditions, the use of ROS in signal transduction can contribute to acclimation and eventually tolerance to various stressors (Hossain et al., 2015).

Among all ROS, H_2O_2 is often put forward as the most attractive signaling molecule (Neill S. et al., 2002; Neill S. J. et al., 2002; Foyer and Noctor, 2005; Petrov and Van Breusegem, 2012). It is produced by a two-step reduction of molecular O_2. Superoxide—generated after the first reduction step—is converted into H_2O_2, for example by superoxide dismutase (SOD). Subsequently, H_2O_2 can give rise to highly toxic $^{\bullet}OH$ radicals through the Fenton and Haber-Weiss reactions with the help of free redox-active metal ions (**Figure 1**; Halliwell, 2006). With a half-life of 1 ms, H_2O_2 is relatively stable as compared to $O_2^{\bullet-}$ and $^{\bullet}OH$ that have a half-life of only 1 μs and 1 ns, respectively (Møller et al., 2007). Additional advantages are its high cellular abundance (up to the low millimolar range) (Cheeseman, 2006; Møller et al., 2007), its small size (Petrov and Van Breusegem, 2012) and its ability to cross cellular membranes through aquaporins and thereby migrate to different cellular compartments (Bienert et al., 2006, 2007; Bienert and Chaumont, 2014). Furthermore, H_2O_2 is an uncharged non-radical with an intermediate oxidation number (−1 for each oxygen atom), implying both oxidizing and reducing properties (Bienert et al., 2007; Bienert and Chaumont, 2014). With regard to H_2O_2 scavenging, it is important to keep in mind the unique property of catalase (CAT) among all antioxidative enzymes: it is able to convert H_2O_2 to H_2O_2 and O_2 without the use of reducing equivalents (see Section "Production and Scavenging of H_2O_2 in Metal-Exposed Plants") (Bienert et al., 2007; Das and Roychoudhury, 2014). The fact that H_2O_2 scavenging by CAT occurs in an energy-efficient way can be a crucial asset under environmental stress conditions, when energy is required to set up an appropriate defense response (Gechev et al., 2006; Das and Roychoudhury, 2014).

Reactive oxygen species are able to transmit a signal by oxidizing a target molecule, for example a transcription factor (Mittler et al., 2004). The relatively long-living H_2O_2 can travel a cellular distance up to 1 μm and brings the signal close to its target, thereby acting as primary messenger. However, the cellular distance traveled by more short-living ROS ranges from a mere nm ($^{\bullet}OH$) up to 30 nm ($^1O_2$ and $O_2^{\bullet-}$). These will therefore react with a cellular compound close to their production site, with the oxidation product acting as second messenger (Møller et al., 2007). However, both routes lead to the same net signaling result for ROS with different physicochemical properties. In the following paragraphs, the production and scavenging of H_2O_2 is discussed in the light of metal stress. Furthermore, results from priming experiments and screenings of metal tolerant vs. sensitive genotypes/ecotypes have revealed a strong relationship between H_2O_2 and metal tolerance in plants.

Production and Scavenging of H_2O_2 in Metal-Exposed Plants

In plants, H_2O_2 and other ROS are continuously produced in different subcellular compartments as byproducts of various metabolic reactions. While most ROS in plant cells originate from chloroplasts and peroxisomes, mitochondria are the most important ROS producers under dark conditions and in non-photosynthetic tissues (Navrot et al., 2007; Das and Roychoudhury, 2014). In chloroplasts and mitochondria, leakage of electrons to O_2 as a consequence of electron transport chain over reduction can generate $O_2^{\bullet-}$ radicals, which can subsequently be converted to H_2O_2. In peroxisomes, H_2O_2 can be directly produced by oxidation reactions of fatty acids and glycolate formed during photorespiration (Petrov and Van Breusegem, 2012).

On the other hand, ROS can also be enzymatically generated in the apoplast. At the plasma membrane, $O_2^{\bullet-}$ is generated by NADPH oxidases. These enzymes are homologs of the mammalian respiratory burst oxidase gp91^{phox} and are therefore referred to as respiratory burst oxidase homologs (RBOHs) (O'Brien et al., 2012). Using NADPH as a cytosolic electron donor, they catalyze the reduction of apoplastic O_2 to $O_2^{\bullet-}$, which can then be dismutated to H_2O_2 either non-enzymatically or by the action of SOD. Furthermore, apoplastic ROS can also be produced by cell wall-anchored class III peroxidases. Although these enzymes are also involved in H_2O_2 scavenging, they are able to generate H_2O_2 in the presence of a strong reductant. Reactive oxygen species produced by the action of these peroxidases play an important role in several developmental processes including cell wall cross-linking and loosening (O'Brien et al., 2012; Kärkönen and Kuchitsu, 2015).

While ROS production in organelles and the apoplast continuously occurs under physiological growth conditions, it can be greatly enhanced by biotic and abiotic stress factors (Gechev et al., 2006; Petrov and Van Breusegem, 2012). As demonstrated in **Table 1**, exposure to even environmentally relevant metal concentrations increased the production of H_2O_2 in a wide variety of plant species. As discussed before, the mechanisms underlying metal-induced ROS production in plants are dependent on the chemical properties of the metal. Indirect metal-induced ROS production can be achieved by several mechanisms (Cuypers et al., 2012). Metals can for example inhibit the activity of various enzymes by binding to their functional groups or by displacement of essential cations in specific binding sites (Gupta et al., 2009; Cuypers et al., 2011). In this way, they can disturb the action of enzymes involved in antioxidative defense and physiological processes such as respiration and photosynthesis, thereby increasing ROS production. Furthermore, metals are able to deplete the pool of the important antioxidant glutathione (GSH), thereby also disturbing the ROS balance (Lee et al., 2003). In addition, several metals were shown to increase ROS production by plasma membrane-bound NADPH oxidases (**Figure 1**; Romero-Puertas et al., 2004; Hao et al., 2006; Remans et al., 2010).

In order to prevent cellular damage as a result of increased ROS production, plants possess an extensive antioxidative defense system consisting of both non-enzymatic and enzymatic compounds (**Figure 1**). Two important non-enzymatic antioxidants are the water-soluble metabolites ascorbate (AsA) and GSH. Ascorbate can directly scavenge $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ radicals and is involved in the regeneration of other antioxidants such as α-tocopherol (Das and Roychoudhury, 2014). Furthermore, it plays an important role in the AsA-GSH cycle. In the first step of this cycle, ascorbate peroxidase (APX) detoxifies H_2O_2 to H_2O using AsA as the reducing agent. Subsequently, the reconversion of AsA to its reduced form is coupled to the oxidation of GSH, which is again reduced by the action of glutathione reductase (GR) (Cuypers et al., 2012). In addition to its involvement in the AsA-GSH cycle, GSH can also directly detoxify ROS and is the substrate of glutathione-S-transferase (GST) enzymes, catalyzing the conjugation of GSH with electrophilic compounds. Plant GSTs are subdivided into several classes and are involved in a wide range of functions including the detoxification of xenobiotics (e.g. herbicides) and products of oxidative DNA and lipid damage (Marrs, 1996; Gill and Tuteja, 2010). Furthermore, GSH plays a role in the scavenging of metals via its sulfhydryl group and is also the precursor of metal-chelating phytochelatins (PCs) (Jozefczak et al., 2012; Noctor et al., 2012). In addition to PCs, also metallothioneins (MTs) are able to bind metals such as Cu, Cd and Zn through the thiol groups of their cysteine residues. Furthermore, several studies suggest that MTs are directly involved in ROS scavenging (Hassinen et al., 2011).

In contrast to the water-soluble AsA and GSH, α-tocopherol and carotenoids are important lipid-soluble antioxidative metabolites. They are involved in protecting membranes against lipid peroxidation and preventing damage to the photosynthetic machinery, respectively (Das and Roychoudhury, 2014). In addition, the amino acid proline accumulates in plants under abiotic stress conditions including metal exposure (Sharma and Dietz, 2006). Proline is able to quench $^1O_2$ and scavenge $^{\bullet}OH$ radicals *in vitro*, and several studies have attributed an antioxidant function to proline under metal stress *in vivo* as well (Sharma and Dietz, 2006). For example, pretreatment of *Oryza sativa* plants with proline decreased the accumulation of H_2O_2 and lipid peroxidation after Hg exposure (Wang et al., 2009). These observations might be related to the fact that proline is able to protect and stabilize ROS scavenging enzymes such as CAT and peroxidases (Sharma and Dietz, 2006; Szabados and Savouré, 2009).

Among the antioxidative enzymes, SODs are responsible for the conversion of $O_2^{\bullet-}$ to O_2 and H_2O_2. Based on the metal present in the active center, these enzymes are classified as Cu/Zn-SOD (localized in the apoplast, cytosol, chloroplasts and peroxisomes), Mn-SOD (localized in mitochondria) or Fe-SOD (localized in chloroplasts) (Alscher et al., 2002; Das and Roychoudhury, 2014). Scavenging of H_2O_2 is performed by CAT, ascorbate peroxidase (APX), glutathione peroxidase (GPX), guaiacol peroxidase, class III peroxidases and peroxiredoxins. In general, peroxidases oxidize a wide range of substrates, thereby reducing peroxides including H_2O_2 (Mathé et al., 2010). While APX reduces H_2O_2 to H_2O using the reducing power of AsA, GPX uses thioredoxin and GSH as electron donors (Das and Roychoudhury, 2014; Bela et al., 2015; Passaia and

TABLE 1 | Metal-induced H_2O_2 production and scavenging in plants.

Metal		Species	H_2O_2 scavenging					References
				APX		CAT		
			H_2O_2 production	Activity	Gene expression	Activity	Gene expression	
Essential	Cu	*Arabidopsis thaliana*	x	x	x	x	x	Cuypers et al., 2011
			x					Liu et al., 2015
			x					Martínez-Peñalver et al., 2012
			x		x		x	Opdenakker et al., 2012a
			x					Yuan et al., 2013
		Cucumis sativus	x			x		İşeri et al., 2011
		Hordeum vulgare	x	x		x		Hu et al., 2015
		Ipomoea batatas	x					Kim et al., 2010
		Matricaria chamomilla	x					Kováčik et al., 2010b
			x	x				Kováčik et al., 2010a
		Medicago truncatula	x					Macovei et al., 2010
		Nicotiana tabacum	x	x				Xia et al., 2012
		Oryza sativa	x	x		x		Mostofa et al., 2015a
			x	x				Thounaojam et al., 2012
		Pauwlonia fortunei	x	x		x		Wang J. et al., 2010
		Silene dioica	x	x				Kováčik et al., 2010b
		Silene vulgaris	x	x				Kováčik et al., 2010b
		Solanum lycopersicum[a]	x	x				İşeri et al., 2011
		Spirodela polyrhiza	x	x		x		Upadhyay and Panda, 2010
	Ni	*Brassica juncea*	x	x				Khan and Khan, 2014
		Brassica napus	x	x		x		Kazemi et al., 2010
		Vicia sativa	x			x		Ivanishchev and Abramova, 2015
	Zn	*Arabidopsis thaliana*	x	x	x	x	x	Remans et al., 2012a
		Brassica juncea	x	x				Feigl et al., 2015
			x	x				Khan and Khan, 2014
		Gossypium hirsutum	x	x		x		Anwaar et al., 2015
		Ipomoea batatas	x					Kim et al., 2010
		Lactuca sativa	x					Barrameda-Medina et al., 2014
		Myracrodruon urundeuva	x	x		x		Gomes et al., 2013
		Pauwlonia fortunei	x	x		x		Wang J. et al., 2010
		Phaseolus vulgaris	x					Michael and Krishnaswamy, 2011
		Populus × canescens	x	x		x		Shi et al., 2015
		Solanum melongena	x	x				Wu et al., 2015
		Solanum nigrum	x	x	x	x	x	Xu Q. S. et al., 2010
		Spirodela polyrhiza	x	x		x		Upadhyay and Panda, 2010
		Verbacum thapsus	x	x				Morina et al., 2010
Non-essential	Al	*Cucumis sativus*	x	x		x		Pereira et al., 2010
		Nicotiana tabacum	x	x				Yin et al., 2010

(Continued)

TABLE 1 | Continued

Metal		Species	H_2O_2 scavenging					References
				APX		CAT		
			H_2O_2 production	Activity	Gene expression	Activity	Gene expression	
Non-essential	Cd	*Arabidopsis thaliana*	x	x	x		x	Cuypers et al., 2011
			x					Martínez-Peñalver et al., 2012
			x			x		Tao et al., 2013
		Boehmeria nivea	x	x				Tang et al., 2015
		Brassica campestris	x					Anjum et al., 2014
		Brassica juncea	x					Masood et al., 2012
		Brassica napus	x	x		x		Ali et al., 2013
		Citrus paradisi × *Poncirus trifoliata*	x			x		Podazza et al., 2012
		Dittrichia viscosa	x	x		x		Fernández et al., 2013
		Glycine max	x	x		x		Pérez-Chaca et al., 2014
		Helianthus annuus	x	x		x		Saidi et al., 2014
		Ipomoea batatas	x					Kim et al., 2010
		Kosteletzkya virginica	x	x		x		Han et al., 2013
		Lactuca sativa	x	x		x		Monteiro et al., 2012
		Lepidium sativum	x	x		x		Gill et al., 2012
		Lupinus luteus	x					Arasimowicz-Jelonek et al., 2012
		Nicotiana tabacum	x	x		x		Iannone et al., 2010
		Oryza sativa	x	x		x		Chou et al., 2011
			x	x		x		Mostofa et al., 2015b
			x			x		Singh and Shah, 2014
			x			x		Srivastava et al., 2014
			x	x				Srivastava et al., 2015
			x					Wang et al., 2014
			x					Yu et al., 2015
		Populus cathayana	x			x		He et al., 2013
		Populus nigra	x	x		x		He et al., 2013
		Populus popularis	x	x				He et al., 2013
		Populus × *canadensis*	x					Di Baccio et al., 2014
		Populus × *canescens*	x	x		x		He et al., 2011
		Sedum alfredii	x			x		Tian et al., 2011
		Solanum lycopersicum	x	x		x		Ahammad et al., 2013
			x	x		x		Monteiro et al., 2011
		Solanum nigrum	x	x		x		Deng et al., 2010
			x	x		x		Liu et al., 2013
		Trigonella foenum-graecum	x	x		x		Zayneb et al., 2015
		Triticum aestivum	x					Moussa and El-Gamal, 2010
		Vigna radiata	x					Anjum et al., 2014
		Zea mays	x					Wahid and Khaliq, 2015
		Zygophyllum fabago	x	x		x		Yildiztugay and Ozfidan-Konakci, 2015
	Hg	*Juncus maritimus*	x	x		x		Anjum et al., 2015
		Medicago sativa	x					Montero-Palmero et al., 2014

(Continued)

TABLE 1 | Continued

Metal		Species	H_2O_2 scavenging				References
				APX		CAT	
			H_2O_2 production	Activity	Gene expression	Activity	Gene expression
Non-essential	Pb	*Arabidopsis thaliana*	x			x	Tao et al., 2013
			x				Yu et al., 2012
		Atractylodes macrocephala	x	x		x	Wang et al., 2013
		Brassica napus	x	x		x	Ali et al., 2014
		Hordeum vulgare	x				Legocka et al., 2015
		Lemna trisulca	x				Samardakiewicz et al., 2015
		Nymphoides peltatum	x			x	Qiao et al., 2013
		Oryza sativa	x			x	Srivastava et al., 2014
		Pauwlonia fortunei	x	x			Wang J. et al., 2010
		Talinum triangulare	x	x		x	Kumar et al., 2013
		Triticum aestivum	x	x		x	Kaur et al., 2013
			x	x		x	Kaur et al., 2015
		Vicia faba	x				Shahid et al., 2012
		Zygophyllum fabago	x	x		x	López-Orenes et al., 2014

[a] *In article as Lycopersicum esculentum.*

Metals have the capacity to induce oxidative stress in plants. An increase in H_2O_2 levels is an indicator of the disturbed redox balance. Plant cells have defense mechanisms to scavenge excess ROS, such as the antioxidative enzymes ascorbate peroxidase (APX) and catalase (CAT). The following table catalogs recent research articles (published since 2010) that reported metal-induced H_2O_2 production. The effects on APX and CAT, at the level of both gene expression and enzymatic activity, are indexed according to essential (Cu, Ni, and Zn) and non-essential metals (Al, Cd, Hg, and Pb) and plant species.

Margis-Pinheiro, 2015). On the other hand, guaiacol peroxidase prefers aromatic compounds such as guaiacol and pyrogallol as electron donors to reduce H_2O_2 (Das and Roychoudhury, 2014). As mentioned before, class III peroxidases can both scavenge and produce ROS. In their regular peroxidative cycle, they catalyze the reduction of H_2O_2 using a variety of electron donors including phenolic compounds, lignin precursors, secondary metabolites and auxins (Mathé et al., 2010; Zipor and Oren-Shamir, 2013). In contrast to the above-mentioned peroxidases, peroxiredoxins detoxify H_2O_2 by oxidizing their own thiol groups, which are back-reduced by the action of thioredoxin, glutaredoxin, cyclophilin or GSH (Tripathi et al., 2009). While GPX, guaiacol peroxidase, class III peroxidases and peroxiredoxins are also involved in other cellular processes, CAT and APX are specifically dedicated to H_2O_2 scavenging and the regulation of redox homeostasis. Therefore, both enzymes are discussed in more detail in this review (**Table 1**). Catalase is a tetrameric heme-containing enzyme catalyzing the detoxification of H_2O_2 to H_2O and O_2, which is mainly present in peroxisomes. The APX enzyme is localized in the cytosol, mitochondria, chloroplasts and peroxisomes and converts H_2O_2 into H_2O during the first step of the AsA-GSH cycle (Das and Roychoudhury, 2014). While APX uses AsA as a reducing agent for H_2O_2 detoxification, the action of CAT does not require any reducing equivalents. This provides plants with an energy-efficient way of H_2O_2 removal, which can be of particular interest under stress conditions (Gechev et al., 2006). However, it is important to note that the affinity of APX for H_2O_2 is much higher than that of CAT (micromolar vs. millimolar range). Therefore, APX is assumed to be mainly involved in the fine-tuning of H_2O_2 detoxification important for its signaling function, while CAT is responsible for the bulk removal of excess H_2O_2 generated during stress conditions (**Figure 2**; Mittler, 2002). As shown in **Table 1**, both H_2O_2 scavenging enzymes are affected at transcriptional and activity level in metal-exposed plants. For example, Cuypers et al. (2011) demonstrated differential effects of Cd and Cu on *CAT* and *APX* gene expression in *Arabidopsis thaliana* plants. Dependent on the metal concentration and isoform considered, expression levels were specifically affected in roots or leaves. Furthermore, expression changes were not always mirrored by the enzyme activities, suggesting that CAT and APX regulation also occurs at the post-transcriptional level under metal stress (Cuypers et al., 2011).

The Link between H_2O_2 and Metal Tolerance in Plants

In recent years, multiple studies have focused on the role of H_2O_2 in plant tolerance to a diverse array of abiotic stress conditions. Research has shown that pretreatment of plants with H_2O_2 can decrease the extent of adverse effects induced by subsequent exposure to abiotic stress factors including salinity, drought, heat, chilling and metals, a phenomenon which is generally referred to as H_2O_2 priming (Hossain et al., 2015). Exposure of plants to low concentrations of H_2O_2 (ranging from 100 to 500 μM) prior to metal treatment was shown to minimize

metal-induced growth reduction, lipid peroxidation, chlorophyll degradation and programmed cell death in different plant species (Chao et al., 2009; Hu et al., 2009; Bai et al., 2011; Xu et al., 2011; Guzel and Terzi, 2013; Yildiz et al., 2013). Heat shock, known to increase H_2O_2 levels, can also induce metal tolerance in plants (Chao et al., 2009; Chou et al., 2012). Even though the mechanisms underlying these observations are not fully elucidated yet, available data so far point to the involvement of metal chelation, antioxidative defense and osmotic regulation in increased metal tolerance.

One of the key players in H_2O_2-induced metal tolerance is GSH. Indeed, many studies demonstrate an elevated GSH level in metal-exposed plants pretreated with H_2O_2 as compared to non-primed plants (Hu et al., 2009; Bai et al., 2011; Xu et al., 2011). As GSH is an important component of the AsA-GSH cycle, the elevated GSH level induced by H_2O_2 pretreatment of metal-exposed plants can contribute to an enhanced H_2O_2 detoxification, thereby reducing the negative effects of metal-induced oxidative stress (Apel and Hirt, 2004). Furthermore, GSH can directly chelate metals, which have a high affinity toward its sulfhydryl group. In addition, GSH is the main constituent of metal-chelating PCs. Metals sequestered by GSH and PCs are transported to the vacuole, decreasing the concentrations of free metal ions in the cytosol and thereby preventing metal-induced damage to cellular macromolecules such as DNA, proteins and membrane lipids. Moreover, vacuolar compartmentalization can also affect the transport of metals from roots to aerial plant parts (Liu W. J. et al., 2010; Jozefczak et al., 2012; Najmanova et al., 2012; Noctor et al., 2012). Indeed, Hu et al. (2009) and Bai et al. (2011) demonstrated a reduced root-to-shoot translocation of Cd in *O. sativa* plants pretreated with H_2O_2. In contrast, Yildiz et al. (2013) showed that H_2O_2 priming increased root-to-shoot translocation of Cr(VI) in *Brassica napus* plants. In these experiments however, H_2O_2 was able to counteract the decrease in fresh weight and the induction of lipid peroxidation caused by subsequent metal exposure. These data suggest that the mechanisms underlying H_2O_2-induced metal tolerance strongly depend on the metal and the plant species under study.

In addition to GSH, other antioxidants also seem to be involved in H_2O_2-induced metal tolerance. Xu et al. (2011) have shown that H_2O_2 priming enhanced the Al-induced increase in AsA levels in root tips of an Al-sensitive *Triticum aestivum* genotype. However, this was not observed in an Al-tolerant genotype, indicating that the inherent plant metal tolerance can influence the effect of exogenous H_2O_2 on the responses to subsequent metal exposure. Besides their levels, also the redox state of GSH and AsA can be affected, as indicated by increases in reduced vs. oxidized metabolite ratios by H_2O_2 priming in root tips of both *T. aestivum* genotypes after Al exposure (Xu et al., 2011).

Besides metabolic antioxidants such as GSH and AsA, also antioxidative enzymes could be involved in H_2O_2 priming. Indeed, several studies demonstrated differences in the activities of antioxidative enzymes such as SOD, CAT and APX between metal-exposed plants that were either primed with H_2O_2 or not (Chao et al., 2009; Hu et al., 2009; Xu et al., 2011; Yildiz et al., 2013). This is either related to the fact that H_2O_2 priming (1) counteracts a metal-induced reduction in antioxidative enzyme activities, probably due to binding of the metal to the protein's cysteine residues or (2) increases basal antioxidative enzyme activities to protect plants from metal-induced oxidative damage. Furthermore, it has been shown that H_2O_2 pretreatment can further stimulate metal-induced increases in the activity of GST (Hu et al., 2009; Bai et al., 2011). Together, these data suggest that H_2O_2 priming reduces the negative consequences of metal exposure, while stimulating the plant's defense mechanisms. This H_2O_2-induced enhancement of antioxidative defense, combined with an increase in metal scavenging, can possibly explain the fact that H_2O_2 priming often diminished metal-induced increases in ROS levels (Hu et al., 2009; Xu et al., 2011; Guzel and Terzi, 2013).

In addition to its effects on metal scavenging and antioxidative defense, other processes were also affected by H_2O_2 priming in metal-exposed plants. A study by Guzel and Terzi (2013) showed that H_2O_2 pretreatment counteracted the reductions in dry matter production, relative water content and water potential in leaves of Cu-exposed *Zea mays*. In addition, H_2O_2 priming reduced the negative effects of Cu on the levels of soluble proteins, sugars, and mineral ions and enhanced the Cu-mediated increase in proline content. These results suggest that the water balance may be a target of H_2O_2 priming in metal-exposed plants (Guzel and Terzi, 2013). Interestingly, proline levels are constitutively enhanced in different metal-tolerant plant species (Sharma and Dietz, 2006). While this may be related to its role in osmoregulation, proline might also confer metal tolerance through its function as metal chelator and ROS scavenger as discussed before (reviewed by Sharma and Dietz, 2006).

It is interesting to note that whereas H_2O_2 priming affects plant responses to metal stress, H_2O_2 alone (without subsequent metal exposure) does not always influence the parameters studied. As mentioned, metal-induced increases in antioxidative enzyme activities are often enhanced by H_2O_2 pretreatment. This does not always imply, however, that the activities of these enzymes are also increased in H_2O_2-primed plants that are not subsequently exposed to metal stress. In a recent review on this topic, Hossain et al. (2015) propose that pretreatment with H_2O_2 induces a mild oxidative challenge activating a ROS-dependent signaling network which results in the accumulation of latent defense proteins including antioxidative enzymes and transcription factors. As a consequence, plants enter a primed state that enables enhanced defense responses upon exposure to subsequent abiotic stressors such as metals.

It has been demonstrated that metal-induced oxidative stress is more powerful in sensitive genotypes or ecotypes (reviewed by Sharma and Dietz, 2009). Among the flowering plants, the metal hyperaccumulating plants *A. halleri*, *Noccaea caerulescens,* and *Alyssum bertolonii* exhibit a greater antioxidative capacity than their sensitive relatives (Sharma and Dietz, 2009). For example, activities of APX and class III peroxidases were highly increased in the Cd and Zn hyperaccumulator *A. halleri* as opposed to its sensitive counterpart *A. thaliana* (Chiang et al., 2006). In addition, CAT activity was more than 500 times higher in roots of the Ni hyperaccumulator *A. bertolonii* as

compared to the non-hyperaccumulator *Nicotiana tabacum*, explaining the higher increase in H_2O_2 levels in the latter after Ni exposure (Boominathan and Doran, 2002). Interestingly, results of different studies on contrasting ecotypes or species indicate that H_2O_2 in particular is a crucial mediator of metal phytotoxicity. Indeed, tolerant and hyperaccumulating plant species often display a constitutively increased level of H_2O_2 scavenging enzymes (Sharma and Dietz, 2009). For example, Cho and Seo (2005) observed a higher survival rate and less lipid peroxidation in Cd-resistant *A. thaliana* mutants as compared to wild-type (WT) plants exposed to 300 or 500 μM Cd, even though the Cd content in the mutants was higher. The decreased Cd sensitivity of the mutants was mainly related to increased activities of several antioxidative enzymes such as APX and GR. Interestingly, the authors did not observe a relation between CAT activity and Cd tolerance. Nevertheless, Cd-resistant mutants had lower H_2O_2 levels as compared to WT plants (Cho and Seo, 2005), again supporting a role for H_2O_2 in plant metal tolerance. Furthermore, ROS production under metal stress could also mediate cross-tolerance to pathogens as reviewed by Poschenrieder et al. (2006). Underlying mechanisms could be the induction of antioxidants and the synthesis of secondary metabolites involved in mechanical defense against pathogen attack (Poschenrieder et al., 2006).

HYDROGEN PEROXIDE MEDIATES DAMAGE AND/OR SIGNALING IN METAL-STRESSED PLANTS

The balance between the generation and removal of ROS affects which reactive oxygen compound is present and at which level. This ultimately determines the extent of oxidative damage and/or signaling (Møller et al., 2007). Indeed, antioxidants function to limit the levels of ROS, thereby enabling them to execute beneficial cellular functions without causing too much damage (Halliwell, 2006). Based mainly on its concentration, but also on its production site and the plant's developmental stage, H_2O_2 affects plant stress responses in two ways (Petrov and Van Breusegem, 2012). In general, high levels of H_2O_2 induce cell death (Gechev and Hille, 2004; Petrov and Van Breusegem, 2012; Petrov et al., 2015). This process is critical during leaf senescence and the hypersensitive response, which are both known to occur in response to different developmental as well as environmental cues (Gechev et al., 2006; Quan et al., 2008; Petrov and Van Breusegem, 2012). At low concentrations, H_2O_2 acts as a signaling molecule by (1) directly affecting the activity of a target molecule involved in signaling or transcription, (2) oxidizing a biological molecule that in its turn acts as second messenger or (3) shifting the cellular redox balance to a more oxidized state (Apel and Hirt, 2004; Petrov and Van Breusegem, 2012). The essential role of H_2O_2 in cellular signaling is underlined by the global transcriptomic analysis of Desikan and coworkers, who demonstrated a H_2O_2-induced change in expression for approximately 1% of all *Arabidopsis* genes represented on the microarray (Desikan et al., 2001). In addition, H_2O_2 is a crucial mediator of plant responses to metal stress as discussed in the following sections.

Ample studies have demonstrated the occurrence of ROS-induced oxidative damage at the molecular level in plants exposed to various metals (**Table 2**). Lipids [especially polyunsaturated fatty acids (PUFAs)], DNA and proteins can be oxidatively damaged by ROS, depending on the reactivity of the latter. Hydrogen peroxide is moderately reactive as compared to other ROS and therefore only directly targets sulfur-containing residues in proteins (Møller et al., 2007). However, H_2O_2 can indirectly contribute to oxidative damage when it—together with $O_2^{\bullet-}$—is converted to highly toxic $^{\bullet}OH$ radicals in the Fenton and Haber-Weiss reactions (**Figure 1**). Hydroxyl radicals are able to abstract a hydrogen atom from PUFA residues in a membrane, thereby initiating lipid peroxidation. The resulting carbon-centered radical quickly reacts with O_2 to produce peroxyl radicals, attacking neighboring PUFA side chains and generating lipid hydroperoxides. These can freely decompose into different reactive species such as aldehydes (e.g. malondialdehyde) and lipid epoxides. Overall, lipid peroxidation leads to increased membrane leakiness and damage to receptors, enzymes and ion channels (Halliwell, 2006). Lipid peroxidation—concomitantly with a rise in H_2O_2/ROS levels—was shown to occur in different plant species exposed to Al (Pereira et al., 2010), Cd (Masood et al., 2012), Cu (Opdenakker et al., 2012a), Hg (Montero-Palmero et al., 2014), Ni (Khan and Khan, 2014), Pb (Kaur et al., 2015), and Zn (Khan and Khan, 2014; **Table 2**). It must be noted that redox-active metals accelerate lipid peroxidation by catalyzing the Fenton and Haber-Weiss reactions and splitting up lipid hydroperoxides into alkoxyl and new $^{\bullet}OH$ radicals to feed the chain reaction (Halliwell, 2006). This was clearly demonstrated by the results of Opdenakker et al. (2012a), comparing H_2O_2 levels and lipid peroxidation in *A. thaliana* plants exposed to either Cu or Cd in a similar setup. Both parameters were more rapidly increased and higher after exposure to the redox-active Cu as opposed to Cd, pointing toward a greater and quicker disturbance of the cellular redox state by the former metal (Opdenakker et al., 2012a). However, plant responses to specific metals must always be interpreted with the applied metal concentration, the duration of exposure, the cultivation system and the considered tissue(s) in mind. Interestingly, oxygenation of PUFAs leads to the production of oxylipins in an enzymatic or non-enzymatic manner (see Section "A Relationship between H_2O_2 and Oxylipins in Metal-Exposed Plants"). As oxylipins mediate plant responses to different stressors (Mithöfer et al., 2004; Dave and Graham, 2012), ROS-induced oxidation of lipids causes the emergence of new signaling molecules (Chmielowska-Bąk et al., 2015).

Although H_2O_2 itself is poorly reactive, different studies have demonstrated oxidative DNA damage and protein oxidation accompanied by an increased H_2O_2 level in various plant species under metal stress (**Table 2**). Oxidative DNA damage is often assessed by the amount of 8-hydroxyguanosine, the most commonly observed ROS-induced modification (Møller et al., 2007). Its levels were increased in Al-exposed *N. tabacum* (Yin et al., 2010) and Cu-treated *Medicago truncatula* plants (Macovei et al., 2010). Moreover, the alkaline comet assay

TABLE 2 | Oxidative damage in plants related to an elevated H_2O_2 content induced by metal exposure.

Metal		Species	Damage						References
			Molecular				Cellular		
			Lipid peroxidation	DNA damage	Protein oxidation	Hallmark genes	Chloroplast	Cell death	
Essential	Cu	*Arabidopsis thaliana*	x						Cuypers et al., 2011
			x						Opdenakker et al., 2012a
			x				x		Martínez-Peñalver et al., 2012
		Cucumis sativus	x						İşeri et al., 2011
		Hordeum vulgare	x				x		Hu et al., 2015
		Matricaria chamomilla	x						Kováčik et al., 2010a,b
		Medicago truncatula		x	x		x		Macovei et al., 2010
		Nicotiana tabacum	x						Xia et al., 2012
		Oryza sativa	x				x		Mostofa et al., 2015a
			x						Thounaojam et al., 2012
		Paulownia fortunei	x				x		Wang J. et al., 2010
		Solanum lycopersicum[a]	x						İşeri et al., 2011
		Spirodela polyrhiza	x				x		Upadhyay and Panda, 2010
	Ni	*Brassica juncea*	x				x		Khan and Khan, 2014
		Brassica napus	x				x		Kazemi et al., 2010
		Chlamydomonas reinhardtii	x				x	x	Zheng et al., 2013
		Vicia sativa	x						Ivanishchev and Abramova, 2015
	Zn	*Brassica juncea*	x				x		Khan and Khan, 2014
		Brassica napus	x					x	Feigl et al., 2015
		Brassica oleracea	x						Barrameda-Medina et al., 2014
		Lactuca sativa	x						Barrameda-Medina et al., 2014
		Myracrodruon urundeuva	x						Gomes et al., 2013
		Oryza sativa	x						Thounaojam et al., 2012
		Paulownia fortunei	x				x		Wang J. et al., 2010
		Phaseolus vulgaris	x						Michael and Krishnaswamy, 2011
		Populus × canescens					x		Shi et al., 2015
		Solanum melongena	x						Wu et al., 2015
		Solanum nigrum	x					x	Xu J. et al., 2010
		Spirodela polyrhiza					x		Upadhyay and Panda, 2010
Non-essential	Al	*Cucumis sativus*	x		x		x		Pereira et al., 2010
		Nicotiana tabacum	x	x					Yin et al., 2010
		Triticum aestivum	x		x				Sun et al., 2015
	Cd	*Arabidopsis thaliana*	x						Cuypers et al., 2011
						x			Keunen et al., 2015
			x				x		Martínez-Peñalver et al., 2012
			x				x	x	Tao et al., 2013

(Continued)

TABLE 2 | Continued

Metal		Species	Damage						References
			Molecular				Cellular		
			Lipid peroxidation	DNA damage	Protein oxidation	Hallmark genes	Chloroplast	Cell death	
Non-essential	Cd	*Boehmeria nivea*	x				x		Tang et al., 2015
		Brassica campestris	x						Anjum et al., 2014
		Brassica juncea	x				x		Masood et al., 2012
		Brassica napus	x				x		Ali et al., 2013
		Citrus paradisi × *Poncirus trifoliata*	x						Podazza et al., 2012
		Dittrichia viscosa	x				x		Fernández et al., 2013
		Glycine max	x		x				Pérez-Chaca et al., 2014
		Helianthus annuus	x						Saidi et al., 2014
		Kosteletzkya virginica	x		x		x		Han et al., 2013
		Lactuca sativa	x	x	x				Monteiro et al., 2012
		Lepidium sativum	x				x	x	Gill et al., 2012
		Lupinus luteus		x				x	Arasimowicz-Jelonek et al., 2012
		Nicotiana tabacum						x	Iannone et al., 2010
		Oryza sativa	x				x		Chou et al., 2011
			x				x		Mostofa et al., 2015b
			x					x	Singh and Shah, 2014
			x		x		x		Srivastava et al., 2014
			x		x			x	Srivastava et al., 2015
			x					x	Yu et al., 2015
		Paulownia fortunei	x				x		Wang J. et al., 2010
		Populus cathayana					x		He et al., 2013
		Populus deltoides					x		He et al., 2013
		Populus × *euramericana*					x		He et al., 2013
		P. alba × *P. glandulosa*					x		He et al., 2013
		Sedum alfredii	x						Tian et al., 2011
		Solanum lycopersicum	x				x		Ahammad et al., 2013
			x				x		Monteiro et al., 2011
		Solanum nigrum	x						Deng et al., 2010
								x	Liu et al., 2013
		Trigonella foenum-graecum	x				x		Zayneb et al., 2015
		Triticum aestivum	x				x		Moussa and El-Gamal, 2010
		Vigna radiata	x						Anjum et al., 2014
		Zea mays	x						Wahid and Khaliq, 2015
		Zygophyllum fabago	x				x		Yildiztugay and Ozfidan-Konakci, 2015
	Hg	*Juncus maritimus*	x		x				Anjum et al., 2015
		Medicago sativa	x						Montero-Palmero et al., 2014
	Pb	*Arabidopsis thaliana*	x				x	x	Tao et al., 2013
		Atractylodes macrocephala	x				x		Wang et al., 2013

(Continued)

TABLE 2 | Continued

Metal		Species	Damage						References
			Molecular				Cellular		
			Lipid peroxidation	DNA damage	Protein oxidation	Hallmark genes	Chloroplast	Cell death	
Non-essential	Pb	*Brassica napus*	x						Ali et al., 2014
		Hordeum vulgare	x				x		Legocka et al., 2015
		Nymphoides peltatum	x				x		Qiao et al., 2013
		Oryza sativa	x		x		x		Srivastava et al., 2014
		Paulownia fortunei	x				x		Wang J. et al., 2010
		Talinum triangulare	x	x	x			x	Kumar et al., 2013
		Triticum aestivum	x					x	Kaur et al., 2013
			x						Kaur et al., 2015
		Vicia faba	x				x		Shahid et al., 2012
		Zygophyllum fabago	x				x		López-Orenes et al., 2014

[a] *In article as Lycopersicum esculentum.*

Exposure to excess metals affects H_2O_2 production and causes molecular and cellular damage in plants. At the molecular level, lipids, DNA and proteins can be oxidized by H_2O_2. Expression of genes that are commonly induced by oxidative stress (Gadjev et al., 2006) can be assessed as marker of metal-induced oxidative damage. Furthermore, damage at the level of the chloroplast and even cell death are often observed under metal stress conditions. The effects of excess essential metals (Cu, Ni, and Zn) as well as non-essential metals (Al, Cd, Hg, and Pb) are shown and categorized based upon the metal and plant species studied. Only recently published papers (starting from 2010) demonstrating a metal-induced rise in H_2O_2 content and damage at molecular and/or cellular level were included in this overview.

revealed DNA damage in roots of Al-exposed *Allium cepa* (Achary et al., 2008), Cd-treated *Lactuca sativa* (Monteiro et al., 2012) and Pb-exposed *Talinum triangulare* plants (Kumar et al., 2013). Although many studies concentrated on DNA oxidation, it is now postulated that RNA is more susceptible to this process. Therefore, targeted RNA oxidation by ROS might be a novel mechanism to post-transcriptionally regulate expression of defense genes (Chmielowska-Bąk et al., 2015).

High intracellular levels of H_2O_2 oxidize both cysteine (-SH) and methionine ($-SCH_3$) residues present in various proteins such as Cu/Zn- and Fe-SOD (Das and Roychoudhury, 2014). Although this may disrupt their enzymatic function and thereby lead to irreversible cell damage, it has been recently postulated to be a way to perceive and further relay a H_2O_2 signal in plant cells (Hardin et al., 2009; Petrov and Van Breusegem, 2012). In addition, protein carbonylation is commonly observed under metal stress (**Table 2**). For example, Al increased the carbonyl protein content in *A. cepa* roots (Achary et al., 2008) and *Cucumis sativus* seedlings (Pereira et al., 2010). Protein carbonyls were significantly enhanced in roots and leaves of *L. sativa* plants after Cd exposure (Monteiro et al., 2012), while similar results were observed in roots and shoots of *O. sativa* seedlings exposed to Pb (Srivastava et al., 2014). Not all proteins are equally sensitive to oxidation (Møller et al., 2007). For example, it has been demonstrated that mainly mitochondrial proteins are oxidized under well-irrigated and drought stress conditions in *T. aestivum* leaves (Bartoli et al., 2004). Moreover, Kristensen et al. (2004) have revealed specific subpopulations of *O. sativa* leaf mitochondrial matrix proteins that were carbonylated after *in vitro* treatment with H_2O_2 or Cu. Again, the possibility exists that ROS-mediated protein oxidation in plant mitochondria (and other compartments) functions as stress indicator, provoking an alarm signal to induce plant responses to developmental as well as environmental changes (Møller and Kristensen, 2004; Møller and Sweetlove, 2010; Chmielowska-Bąk et al., 2015). In conclusion, various oxidatively modified molecules serve as signaling compounds, supporting the view that oxidative damage and signaling are two sides of the same coin (Møller et al., 2007). Providing experimental evidence for this hypothesis during metal stress is an intriguing research challenge for the future.

In addition to damage at the molecular level, metal-exposed plants also suffer from (sub)cellular damage. This is often visible at the chloroplast level, leading to inhibition of photosynthesis (**Table 2**; Cuypers et al., 2009). Chlorophyll content was decreased in various plant species exposed to Al (Pereira et al., 2010), Cd (Zawoznik et al., 2007), Cu (Hu et al., 2015), Ni (Kazemi et al., 2010), Pb (Legocka et al., 2015), and Zn (Khan and Khan, 2014). In addition, different photosynthetic parameters (e.g. net photosynthesis rate) were reduced in *A. thaliana* plants exposed to Cd or Pb (Tao et al., 2013). Levels of H_2O_2 were significantly increased after metal exposure in all of the above-mentioned studies, pointing toward a correlation between H_2O_2 and the observed effects at the chlorophyll/photosynthesis level. In addition to chloroplast function and morphology, Cd exposure disturbed the distribution and mobility of mitochondria in *A. thaliana* protoplasts (Bi et al., 2009). Finally, it is important to note that metals are able to initiate H_2O_2-induced programmed cell death (**Table 2**). In Cd-exposed *N. tabacum* cells, NADPH oxidase was activated by a rise in cytosolic free Ca^{2+} concentrations, leading to H_2O_2 production and cell death (Garnier et al., 2006). Cadmium was also shown to increase the production of H_2O_2, which preceded cell death in *A. thaliana* cell suspension cultures (De Michele et al., 2009). Similarly, other studies indicate a relationship between metal exposure, oxidative stress and cell death using roots, root tips or leaf disks and different techniques to assess cell viability (**Table 2**; Pan et al., 2001; Achary et al., 2008; Iannone et al., 2010; Arasimowicz-Jelonek et al., 2012;

Kumar et al., 2013; Feigl et al., 2015). Reactive oxygen species and H_2O_2 in particular are considered as crucial signals that modulate (programmed) cell death in plants (Gechev and Hille, 2004; Gadjev et al., 2008; Petrov et al., 2015), again highlighting the intimate relationship between ROS-mediated damage and signaling (**Figure 2**).

HYDROGEN PEROXIDE DIRECTLY MEDIATES METAL-INDUCED OXIDATIVE SIGNALING

The use of ROS as signaling molecules offers various potential advantages as discussed by Mittler et al. (2011). Their levels can rapidly change by shifting the balance between production and scavenging, which are both tightly controlled in space because of the presence of pro- and anti-oxidative enzymes at different subcellular locations (Mittler et al., 2004). The different molecular properties of various ROS offer the potential to transmit specific signals, also with regard to second messenger products formed after oxidative modification. Signaling is possible both within and across cells, generating a so-called ROS "wave" (Mittler et al., 2011; Baxter et al., 2014). Finally, ROS signaling integrates with several other signaling molecules and mechanisms such as Ca^{2+} and protein phosphorylation. In addition, ROS are directly linked to the plant's cellular homeostasis and metabolism. Therefore, they are perfectly suited to signal any metabolic change occurring during developmental and environmental stimuli (Mittler et al., 2011; Baxter et al., 2014).

Foyer and Noctor (2005) have described ROS-induced signaling through a "ripple" or domino effect over space and/or time, starting with a localized and/or transient oxidative burst affecting the expression of defense and regulatory genes in a transient or more sustained manner. Indeed, ROS are shown to activate various signaling compounds such as kinases/phosphatases, metabolites and hormones, which in their turn affect the expression of different target genes. This finally triggers acclimation to the altered developmental or environmental conditions a plant is experiencing (Mittler et al., 2004; Bienert and Chaumont, 2014). Particularly with regard to H_2O_2, it is interesting to note that it is produced in response to a wide variety of internal and external stimuli and therefore potentially contributes to cross-tolerance toward various stressors (Neill S. J. et al., 2002; Perez and Brown, 2014). Although oxidative stress commonly occurs in various stress conditions, the underlying signaling mechanisms may be highly stress-specific. This is underlined by the identification of marker transcripts specifically regulated by $^1O_2$, $O_2^{\bullet -}$ or H_2O_2 after exposure to different oxidative stress-causing agents. However, several transcripts were classified as general oxidative stress response markers because they responded to most of the applied treatments (Gadjev et al., 2006) and were also induced by Cd stress (Keunen et al., 2015; **Table 2**). Interestingly, Sewelam et al. (2014) have shown that H_2O_2 originating specifically from either chloroplasts or peroxisomes did have a differential impact on the *A. thaliana* transcriptome. Specificity of ROS-induced signaling might be related to the ROS type, amount, source and subcellular location of production, as well as their perception by different sensors (Miller et al., 2008; Cuypers et al., 2012).

Perception of H_2O_2 during Metal Stress

Researchers have long been puzzled by the mechanism(s) used by plants to perceive stress-induced increases in H_2O_2 production and to relay this signal. A minimum of three potential mechanisms has been described: (1) H_2O_2 receptors that remain unidentified to date, (2) redox-sensitive transcription factors and (3) ROS-mediated inhibition of phosphatases (Mittler et al., 2004; Miller et al., 2008). Currently, it is still assumed that redox-sensitive transcription factors are oxidized by H_2O_2 and directly activate downstream signaling cascades (Neill S. et al., 2002; Miller and Mittler, 2006; Dietz, 2014). For example, class A heat shock factors (HSFs) are known to respond to oxidative stress in animals and plants (Petrov and Van Breusegem, 2012). The potential involvement of HSFs in perceiving H_2O_2 during metal stress (Miller and Mittler, 2006) is supported by the observed production of heat shock proteins in various metal-exposed plants (di Toppi and Gabbrielli, 1999; Cuypers et al., 2009). Miller et al. (2008) have proposed a model for ROS signaling using plants that lack the cytosolic APX1 isoform. In this model, different HSFs function as H_2O_2 sensors upstream of other transcription factors of the zinc finger protein ZAT (ZAT7, 10 and 12) and WRKY family (e.g. WRKY25) (Miller et al., 2008). Interestingly, expression levels of *ZAT12* and *WRKY25* genes were induced in *A. thaliana* plants exposed to Cd or Cu (Opdenakker et al., 2012a). Both genes were more rapidly induced upon exposure to Cu than to Cd in the roots, corresponding with the observed differences in H_2O_2 levels and potentially related to the contrasting redox properties of both metals (Opdenakker et al., 2012a).

A central protein involved in ROS sensing is the serine/threonine protein kinase oxidative signal-inducible 1 (OXI1). This enzyme is directly induced by H_2O_2 and forms an essential part of the signal transduction pathway linking ROS production to diverse downstream responses (Rentel et al., 2004). It also connects redox to lipid signaling via phosphatidic acid in a phosphoinositide-dependent kinase (PDK1)-related manner (Anthony et al., 2004, 2006). Interestingly, Opdenakker et al. (2012a) demonstrated highly increased *OXI1* transcription in Cd- or Cu-exposed *A. thaliana* plants. Again, its upregulation was higher and earlier induced after exposure to Cu, potentially related to its redox-active properties. Results by Smeets et al. (2013) underscore the key role of OXI1 in cellular signaling responses to Cu stress using *oxi1* knockout *A. thaliana* mutants. As compared to WT plants, plants lacking OXI1 responded differently to redox-induced changes (Smeets et al., 2013). Downstream of OXI1, mitogen-activated protein kinases (MAPKs) control the activation of multiple defense mechanisms in response to oxidative stress as discussed in the following section.

Hydrogen Peroxide Signal Transduction by MAPKs and Transcription Factors

One of the typical downstream signaling events associated with H_2O_2 sensing is the activation of MAPK pathways (**Table 3**;

TABLE 3 | Signaling responses related to an elevated H_2O_2 content induced by metal exposure.

Metal		Species	TFs	MAPKs	Phytohormones	References
Essential	Cu	*Arabidopsis thaliana*	WRKY, ZAT	MPK3/6		Opdenakker et al., 2012a
					Aux	Yuan et al., 2013
		Oryza sativa			JA[a]	Mostofa et al., 2015a
		Spirodela polyrhiza			JA[a]	Upadhyay and Panda, 2010
	Ni	*Brassica juncea*			Eth	Khan and Khan, 2014
	Zn	*Brassica juncea*			Eth	Khan and Khan, 2014
		Brassica oleracea			JA[a]	Barrameda-Medina et al., 2014
		Lactuca sativa			JA[a]	Barrameda-Medina et al., 2014
		Populus × canescens			ABA, SA	Shi et al., 2015
		Solanum melongena			ABA, Aux, CK	Wu et al., 2015
Non-essential	Cd	*Arabidopsis thaliana*		MPK3/6		Liu X. M. et al., 2010
			WRKY, ZAT	MPK3/6		Opdenakker et al., 2012a
					JA[a]	Remans et al., 2010
					JA	Keunen et al., 2013
					SA	Tao et al., 2013
		Brassica juncea			Eth	Masood et al., 2012
		Citrus paradisi × Poncirus trifoliata			JA[a]	Podazza et al., 2012
		Kosteletzkya virginica			Aux, CK, Eth, SA	Han et al., 2013
		Lupinus luteus			SA	Arasimowicz-Jelonek et al., 2012
		Oryza sativa			JA[a]	Mostofa et al., 2015b
					Aux	Yu et al., 2015
		Triticum aestivum			ABA	Moussa and El-Gamal, 2010
	Hg	*Medicago sativa*			Eth	Montero-Palmero et al., 2014
	Pb	*Arabidopsis thaliana*			SA	Tao et al., 2013
		Zygophyllum fabago			SA	López-Orenes et al., 2014

[a] *Solely reported as an effect on LOX gene expression or LOX activity in article.*

During metal stress, several signaling responses are induced by increased H_2O_2 levels. Several transcription factors (TFs) and MAPKs and are activated by H_2O_2. In addition, multiple phytohormone signaling pathways are affected by different metals. The effects of excess essential metals (Cu, Ni, and Zn) as well as non-essential metals (Al, Cd, Hg, and Pb) are shown and categorized based upon the metal and plant species studied. Only recently published papers (starting from 2010) demonstrating a metal-induced rise in H_2O_2 content and signaling were included in this overview. Abbreviations: ABA, abscisic acid; Aux, auxins; CK, cytokinin; Eth, ethylene; JA, jasmonic acid; SA, salicylic acid.

Mittler et al., 2004; Colcombet and Hirt, 2008). These signaling modules are found in all eukaryotic cells and consist of at least three kinases (MAP3K, MAP2K and MAPK) specifically phosphorylating and thereby activating each other (Colcombet and Hirt, 2008; Opdenakker et al., 2012b). Several authors have reported the involvement of MAPK signaling during exposure to Cd, Cu, Hg, Pb and Zn in different plant species (Opdenakker et al., 2012b and references therein). Upstream of MAPKs, the OXI1 kinase is considered to be a central player in metal-induced oxidative stress responses. Rentel et al. (2004) have shown that the activation of the MAPK isoforms MPK3 and MPK6 by H_2O_2 is reduced in *A. thaliana* plants lacking OXI1. Concurrently with *OXI1*, expression levels of its targets *MPK3* and *MPK6* were enhanced in Cd- or Cu-exposed *A. thaliana* plants (Opdenakker et al., 2012a). Jonak et al. (2004) studied the kinetics of different MAPK activities after exposure to either Cd or Cu in *M. sativa* seedlings. Similar to the results at the transcript level (Opdenakker et al., 2012a), Cu ions rapidly activated these enzymes while Cd exposure led to a delayed stimulation (Jonak et al., 2004). Since GSH effectively inhibited MPK3 and MPK6 activation in Cd-exposed *A. thaliana* plants, H_2O_2/ROS were shown to play a crucial role in this process (Liu X. M. et al., 2010).

In addition to OXI1, also the MAP3K *Arabidopsis* NPK1-like protein kinase 1 (ANP1) is directly activated by H_2O_2 and initiates a phosphorylation cascade via MPK3 and MPK6 (Kovtun et al., 2000). Expression levels of *ANP1* were increased in roots of Cu-exposed *A. thaliana* plants after 6 and 24 h (Opdenakker et al., 2012a). Although MAPKs can be activated by H_2O_2, they also trigger an H_2O_2-mediated oxidative burst themselves (Mittler et al., 2004; Petrov and Van Breusegem, 2012). Indeed, MEK2 (the *Nicotiana* ortholog of *Arabidopsis* MKK4/5) was implicated in ROS production upon fungal infection in *N. benthamiana* by acting upstream of *RBOH* genes known to evoke H_2O_2 production (Yoshioka et al., 2003). Similarly, expression of constitutively active MKK4/5 led to H_2O_2 generation and cell death in *A. thaliana* (Ren et al., 2002). As MAPK cascades function both up- and downstream of H_2O_2 (Mittler et al., 2004; Pitzschke and Hirt, 2006; Pitzschke et al., 2009; Petrov and Van Breusegem, 2012), the existence of positive feedback loops between H_2O_2 and MAPKs

such as MKK4/5 deserves further attention under metal stress conditions.

Activated MAPK cascades are able to regulate downstream gene expression by activating or repressing transcription factors (Colcombet and Hirt, 2008). Transcription factors of the ZAT, WRKY, NAC, DREB, bZIP and MYB family therefore constitute the final link in the signaling chain induced by H_2O_2 (Petrov and Van Breusegem, 2012). Results by Pitzschke et al. (2009) have demonstrated the involvement of a complete MAPK cascade consisting of MEKK1, MKK1/MKK2, and MPK4 in regulating ROS-induced stress signaling. Indeed, the majority of transcription factors responsive to multiple ROS-producing conditions are controlled by this pathway (Pitzschke et al., 2009). Furthermore, MEKK1 is able to directly interact with and phosphorylate the transcription factor WRKY53 (Miao et al., 2007), which could be involved in metal-induced senescence (see Section "Metal-Induced Responses at the Cellular Level: is H_2O_2 Involved in Root Growth Inhibition and Senescence?").

Different members of the ZAT family of zinc finger transcription factors were strongly induced by ROS at the transcript level (Gadjev et al., 2006). In particular, isoforms 7, 10 and 12 have been put forward to be involved in ROS signaling during abiotic stress (Davletova et al., 2005a; Miller et al., 2008). In addition, WRKY transcription factors could function up- or downstream of ZAT proteins (Miller et al., 2008). The WRKY proteins, belonging to one of the largest transcription factor families in plants (Eulgem and Somssich, 2007), all contain the invariable WRKY amino acid signature and recognize W-box *cis* elements in target gene promoter regions. The induction of WRKY25 during oxidative stress was shown to be ZAT12-dependent (Rizhsky et al., 2004). As mentioned before, both *ZAT12* and *WRKY25* expression was induced in Cd- or Cu-exposed *A. thaliana* plants (Opdenakker et al., 2012a), further supporting their involvement in metal-induced ROS signaling. For members of the NAC, DREB, bZIP and MYB family associated with H_2O_2 signaling, their relation to metal stress is to our knowledge generally unexplored to date. Nevertheless, several NAC transcription factors were shown to be H_2O_2-responsive (Balazadeh et al., 2010) and govern leaf senescence in *A. thaliana* (Balazadeh et al., 2008). As discussed in the Section "Metal-Induced Responses at the Cellular Level: Is H_2O_2 Involved in Root Growth Inhibition and Senescence?," metal exposure might induce a hastening of this naturally occurring process and the role of NAC transcription factors herein might be an interesting topic for future research. This is further supported by promising results of Fang and coworkers, who recently demonstrated the stress-responsive SNAC3 transcription factor to confer tolerance to heat and drought stress in *O. sativa* plants by modulating ROS (Fang et al., 2015).

Although OXI1, MPK3 and MPK6 were shown to be activated in metal-exposed plants, information on upstream signaling pathways as well as downstream targets under metal stress conditions is rather scarce. Nevertheless, defined end points of specific MAPK signaling pathways are critical to activate the plant's antioxidative defense during metal-induced oxidative stress (Cuypers et al., 2012). In response to H_2O_2, MAPK regulation of ZAT12 led to enhanced expression of the *APX1* gene in *A. thaliana* (Rizhsky et al., 2004). This gene, encoding a cytosolic H_2O_2 scavenging enzyme, was shown to protect the chloroplast redox state during light stress (Davletova et al., 2005b). Interestingly, also the *CAT1* gene was shown to be regulated by MAPK signaling in *A. thaliana* (Xing et al., 2007, 2008). Both *APX1* and *CAT1* are critical in scavenging metal-induced H_2O_2 and were induced in *A. thaliana* plants exposed to Cd, Cu, or Zn (**Table 1**; Cuypers et al., 2011; Remans et al., 2012a). Interestingly, Davletova et al. (2005b) have postulated the involvement of MAPK-regulated *RBOHD* expression in ROS signal amplification during light stress, and further studies confirmed its role in abiotic stress-induced systemic signaling (Miller et al., 2009). Expression of *RBOHD* was also induced upon Cd, Cu and Zn exposure in *A. thaliana* (Remans et al., 2010, 2012a; Cuypers et al., 2011). Although all of the above-mentioned components have been separately assessed under metal stress conditions, further efforts should be made to reveal the sequence of events from stress perception to response in metal-exposed plants.

Metal-induced MAPK signaling pathways show extensive crosstalk with phytohormone signaling. Upon activation, both MPK3 and MPK6 can phosphorylate 1-aminocyclopropane-1-carboxylate synthase (ACS) isoforms 2 and 6, increasing their half-life and the production of ethylene by these enzymes (Liu and Zhang, 2004; Joo et al., 2008; Han et al., 2010). Transcription of both ACS isoforms can also be enhanced by MPK3/6 via the WRKY33 transcription factor (Li et al., 2012). In addition, Yoo et al. (2008) have shown that a MKK9-MPK3/6 cascade promotes ethylene signaling by phosphorylating the nuclear transcription factor ethylene-insensitive 3 (EIN3) in *A. thaliana*. Increasing evidence supports a role for ethylene in regulating metal stress responses in plants (reviewed by Thao et al., 2015; Keunen et al., 2016). It has been demonstrated that the increase in ethylene levels was mainly related to upregulated *ACS2* and *ACS6* expression in Cd-exposed *A. thaliana* plants (Schellingen et al., 2014). Furthermore, MPK3 and MPK6 were proposed to connect ROS production to ethylene signaling in *A. thaliana* leaves under Cd exposure. Cadmium activates NADPH oxidases that produce ROS, which are sensed by OXI1. This kinase then activates MPK3 and MPK6, both affecting ACS2 and ACS6 enzymes at various levels (Schellingen et al., 2015). In conclusion, ethylene shows extensive crosstalk with signaling by ROS or H_2O_2 under metal stress (Thao et al., 2015; Keunen et al., 2016), which should definitely be explored in more detail in future studies. Also the production of other phytohormones such as abscisic acid (ABA), auxins, cytokinins, jasmonic acid (JA) and salicylic acid (SA) is affected by metal exposure in different plant species (**Table 3**). Compelling evidence for a role of endogenous SA in Pb and Cd tolerance of *A. thaliana* was provided by Tao et al. (2013). Metal-induced phytotoxicity was potentiated by elevating endogenous SA levels, while plants with lower SA levels performed better when exposed to Pb or Cd. One of the underlying mechanisms of SA-mediated toxicity is related to plant redox homeostasis, with SA-accumulating plants showing higher metal-induced H_2O_2 concentrations as compared to SA-deficient plants (Tao et al., 2013). As discussed by Petrov and Van Breusegem (2012), interactions between H_2O_2 and SA can range from cooperation

to inhibition depending on the used experimental conditions. Therefore, much work remains to be done to fully unravel the interaction between H_2O_2 and phytohormones such as ethylene and SA during metal stress in plants. In addition, a link between H_2O_2 and JA in metal-exposed plants is evident and discussed in the Section "A Relationship between H_2O_2 and Oxylipins in Metal-Exposed Plants".

HYDROGEN PEROXIDE INTERACTS WITH OTHER SIGNALING PATHWAYS AND REGULATING MECHANISMS

As mentioned before, H_2O_2 is connected to a variety of signaling molecules (e.g. MAPK) and plant hormones (e.g. ethylene). In this section, we discuss its relation to Ca^{2+}, nitric oxide ($NO^{\bullet}$), oxylipins and microRNAs in general and demonstrate evidence for their involvement during the metal-induced oxidative challenge in plants (**Figure 2**).

Interaction between H_2O_2 and Ca^{2+} in Metal-Exposed Plants

Compelling evidence indicates a reciprocal relationship between H_2O_2 and Ca^{2+}, two crucial messengers involved in plant responses to multiple stress conditions (Tuteja and Mahajan, 2007; Quan et al., 2008; Mazars et al., 2010; Petrov and Van Breusegem, 2012). Rentel and Knight (2004) observed a biphasic increase in cytosolic Ca^{2+} levels of *Arabidopsis* seedlings upon treatment with H_2O_2. Enhancing or reducing the height of the Ca^{2+} peaks had a corresponding effect on the expression of the H_2O_2-responsive *GST1* gene, indicating crosstalk between H_2O_2 and Ca^{2+} signaling in plants (Rentel and Knight, 2004). Whereas ROS modulate cytosolic Ca^{2+} levels through the activation of Ca^{2+} channels in the plasma membrane, H_2O_2 production by NADPH oxidases reversely depends on Ca^{2+} (reviewed by Mazars et al., 2010). In Cd-exposed bright yellow-2 *N. tabacum* cells, H_2O_2 production was preceded by an enhanced cytosolic Ca^{2+} level essential to activate NADPH oxidases (Garnier et al., 2006). Indeed, Ca^{2+} directly binds EF-hand motifs in the cytosolic N-terminal domain of the NADPH oxidase enzyme and leads to phosphorylation of the N-terminus by activating a calcium-dependent protein kinase (CDPK) (Sagi and Fluhr, 2006; Kobayashi et al., 2007; Ogasawara et al., 2008). The potential involvement of CDPK in metal stress responses is supported by the transcriptional induction of the *CDPK1* gene in roots of Cd-exposed *A. thaliana* plants (Smeets et al., 2013). Furthermore, several CDPK isoforms in *T. aestivum* were responsive to H_2O_2 treatment, indicating a role for these enzymes in oxidative signaling in plants (Li et al., 2008; Schulz et al., 2013). Interestingly, an increased Ca^{2+} concentration in peroxisomes caused by elevated cytosolic Ca^{2+} levels was shown to stimulate CAT3 activity *in vivo*. The resulting rise in peroxisomal H_2O_2 scavenging potential (Costa et al., 2010) could also be important during metal-induced oxidative stress. In this regard, the cellular response of *Pisum sativum* plants to long-term Cd exposure was shown to involve extensive crosstalk between Ca^{2+}, ROS and $NO^{\bullet}$ (Rodríguez-Serrano et al., 2009) as discussed in the following section. Finally, Baliardini et al. (2015) recently reported a positive correlation between the expression of a gene encoding a Ca^{2+}/H^{+} exchanger (*CAX1*) and Cd tolerance in *Arabidopsis*. Indeed, its expression was higher in the Cd-tolerant *A. halleri* as compared to its Cd-sensitive relative species *A. lyrata* and *A. thaliana*. Plants without functional CAX1 also show increased accumulation of H_2O_2 when exposed to Cd, suggesting a role for CAX1 in maintaining cytosolic Ca^{2+} levels and thereby avoid uncontrolled ROS accumulation during oxidative stress conditions (Baliardini et al., 2015).

Nitric Oxide and H_2O_2: Friends or Foes during Metal Exposure?

Nitric oxide ($NO^{\bullet}$) production is often induced by abiotic stress in plants, for example during exposure to different metals (reviewed by Xiong et al., 2010). In contrast, *P. sativum* plants showed reduced $NO^{\bullet}$ levels under long-term (14 days) Cd exposure (Rodríguez-Serrano et al., 2009). The authors hypothesized, since $NO^{\bullet}$ is able to react with $O_2^{\bullet-}$, that these lower $NO^{\bullet}$ levels could result in $O_2^{\bullet-}$ accumulation under Cd stress. This was further supported by decreased $O_2^{\bullet-}$ levels when $NO^{\bullet}$ production was restored in Cd-exposed plants by application of additional Ca (Rodríguez-Serrano et al., 2009). Different authors have reported the potential of exogenous $NO^{\bullet}$ to alleviate metal toxicity in plants (Xiong et al., 2010). For example, it has been proposed that $NO^{\bullet}$-induced Cu tolerance in *Lycopersicon esculentum* plants was mediated by H_2O_2 detoxification and the accumulation of Cu-scavenging metallothioneins (Wang L. et al., 2010). Although external application of $NO^{\bullet}$ activated the antioxidative defense system, endogenous $NO^{\bullet}$ could also contribute to metal phytotoxicity (reviewed by Arasimowicz-Jelonek et al., 2011). For example, $NO^{\bullet}$ is known to promote the upregulation of genes involved in Fe uptake under Cd stress, thereby also contributing to increased Cd uptake in *A. thaliana* (Besson-Bard and Wendehenne, 2009; Besson-Bard et al., 2009). On the other hand, it is proposed that $NO^{\bullet}$ produced by plants challenged with low Cd concentrations could mediate signaling responses leading toward metal tolerance (Arasimowicz-Jelonek et al., 2011). It is clear that further research is required to fully unravel the role of $NO^{\bullet}$ and its interaction with H_2O_2 and oxidative stress (Petrov and Van Breusegem, 2012) during metal exposure in plants.

A Relationship between H_2O_2 and Oxylipins in Metal-Exposed Plants

Various stress stimuli, such as exposure to different metals, activate biosynthetic enzymes responsible for the accumulation of oxylipins. These are derived from the oxidation of PUFAs by lipoxygenase (LOX) enzymes, with the phytohormone JA and its volatile derivative methyl jasmonate (MeJA) often considered to be the most important in signaling (Browse, 2009; Dave and Graham, 2012; Santino et al., 2013; Wasternack and Hause, 2013). In addition, a non-enzymatic route triggered by ROS is responsible for the synthesis of phytoprostane oxylipins that are also involved in plant stress responses (Dave and Graham, 2012). Evidence for a role of oxylipins during metal stress is

provided by the observed induction of LOX at the transcript and activity level in various plant species (**Table 3**; Skórzyńska-Polit et al., 2006; Tamás et al., 2009; Remans et al., 2010; Keunen et al., 2013; Barrameda-Medina et al., 2014). Furthermore, JA levels increased in *A. thaliana* and *Phaseolus coccineus* plants exposed to Cd or Cu (Maksymiec et al., 2005), supporting a role for JA signaling in mediating stress responses in metal-exposed plants (Maksymiec, 2007). For example, MeJA was shown to upregulate the same set of genes involved in GSH biosynthesis that were also induced in Cd- or Cu-exposed *A. thaliana* plants (Xiang and Oliver, 1998). Interestingly, exogenously applied MeJA induced H_2O_2 production, lipid peroxidation and LOX activity in *Taxus chinensis* cells (Wang and Wu, 2005). Similarly, application of MeJA to *A. thaliana* roots strongly increased H_2O_2 concentrations in the leaves (Maksymiec and Krupa, 2002). This points toward a link between both JA and H_2O_2, suggesting that JA may contribute to metal-induced oxidative stress responses in plants (Rodríguez-Serrano et al., 2009).

MicroRNAs and Redox Signaling in Metal-Exposed Plants

Together with small interfering RNAs (siRNAs), microRNAs (miRNAs) are endogenous non-coding small RNAs involved in the regulation of plant development and stress responses (Vazquez et al., 2010). MicroRNAs negatively regulate their target genes by (1) mRNA cleavage or inhibition of translation or (2) DNA methylation. Expression of different miRNAs is affected by metal stress in different plant species (reviewed by Gielen et al., 2012; Gupta et al., 2014). In general, miRNA-mediated responses are related to metal complexation, antioxidative defense and stress signaling. For example, miR395 regulates sulfate assimilation and was induced in Cd-exposed *B. napus* seedlings (Huang et al., 2010). Sulfate assimilation into cysteine is ultimately required to synthesize GSH and PCs able to chelate free Cd ions, suggesting a role for miR395 in regulating Cd complexation in plants (Gielen et al., 2012). In *Arabidopsis*, *miR398* expression is downregulated by excess Cu, resulting in transcriptional induction of its target genes *Cu/Zn-SOD 1* and *2* (*CSD1*/2). As compared to Cu, Cd exposure oppositely affected both *miRNA398* and *CSD1/2* expression levels, indicating metal-specific regulation potentially related to the redox-active vs. non-redox-active nature of Cu vs. Cd (Cuypers et al., 2011). Interestingly, Cu exposure did not reduce *miR398* expression in leaves of *A. thaliana* plants lacking functional OXI1 as it did in WT plants, pointing toward an interaction between miR398 and MAPK signaling during metal stress (Smeets et al., 2013). Finally, various target genes of metal-induced miRNAs are involved in phytohormone biosynthesis and signaling, often by affecting transcription factors (Gielen et al., 2012; Gupta et al., 2014). Panda and Sunkar (2015) have recently discussed the potential role of redox signaling and/or ROS in inducing stress-responsive miRNAs in plants. This is further supported by a genome-wide study in *O. sativa*, showing seven miRNA families to be induced or downregulated by H_2O_2 treatment (Li et al., 2011). One of the miRNAs upregulated by H_2O_2 is *miR397*, targeting laccase enzymes involved in lignin biosynthesis. Interestingly, metal exposure was also shown to induce *miR397* (reviewed by Gielen et al., 2012; Gupta et al., 2014), suggesting a potential role for H_2O_2 in mediating this induction under metal stress conditions. Future studies should aim to unravel the interplay between metal-induced production of ROS/H_2O_2 and its effects on the induction or downregulation of specific miRNAs targeting downstream response genes.

METAL-INDUCED RESPONSES AT THE CELLULAR LEVEL: IS H_2O_2 INVOLVED IN ROOT GROWTH INHIBITION AND SENESCENCE?

As indicated in **Tables 1**–**3**, metal exposure increases H_2O_2 levels in a variety of plant species, thereby inducing both oxidative damage and signaling responses. At the cellular level, this might underlie metal-induced responses observed in roots (e.g. growth inhibition) and leaves (e.g. premature senescence). For example, Cd-induced oxidative stress could be related to the inhibition of root initiation and elongation (Lux et al., 2011). However, also plant hormones might regulate root growth of metal-exposed plants (Remans et al., 2012b; De Smet et al., 2015). As ROS are shown to interact with phytohormones such as ethylene, future research efforts should be made to dissect their role as potential modulators of root development under metal stress conditions.

Many of the parameters listed in **Table 2** (e.g. lipid peroxidation) can also be regarded as indicators of plant senescence. Indeed, it is known that plants exposed to metals such as Cu and Cd show an accelerated appearance of senescence symptoms (Maksymiec, 2007). During the senescence process, leaves are degraded in a highly regulated fashion in order to remobilize nutrients to developing plant tissues. Leaf senescence comprises the final stage of leaf development and its onset is determined by the developmental age of leaves (Lim et al., 2007). It has been shown, however, that this process can be prematurely induced by several biotic and abiotic stress factors such as pathogen attack, wounding, darkness, drought, salinity, UV-B irradiation and ozone (Miller et al., 1999; John et al., 2001; Espinoza et al., 2007; Zhou et al., 2011; Guo and Gan, 2012; Allu et al., 2014; Zhou et al., 2014).

An important characteristic of senescence is the degradation of cellular macromolecules such as chlorophyll, lipids, proteins and nucleic acids. During the end stage of senescence, cells undergo programmed cell death (Lim et al., 2007). As shown in **Table 2**, many of these features are also affected by metal exposure in plants. In addition, it is known that several components of metal-induced signaling responses are also key players in the initiation and regulation of the senescence process. For example, changes in phytohormone levels are known to affect the onset of leaf senescence. While cytokinins, gibberellins and auxins delay the appearance of senescence symptoms, increases in the levels of other phytohormones such as ethylene, ABA, JA and SA have been shown to accelerate the process (Lim et al., 2007; Fischer, 2012).

Furthermore, transcriptional regulation mechanisms also play an important role in leaf senescence. In *A. thaliana* leaves, for example, more than 800 genes are differentially expressed during senescence (Buchanan-Wollaston et al., 2005). While certain genes such as those encoding photosynthetic proteins are transcriptionally downregulated, the expression of many other genes significantly increases when leaves enter the senescent stage. The latter genes are generally termed "senescence-associated genes" or SAGs and encode proteins involved in the breakdown of cellular compounds (e.g. nucleases, proteases and cell wall hydrolases) and the remobilization of nutrients to developing plant tissues. Also numerous transcription factors, many of which belong to the NAC and WRKY transcription factor families, are considered as SAGs (Miao et al., 2004; Fischer, 2012). For example, overexpression of the NAC transcription factor ORESARA1 SISTER1 (ORS1) accelerates senescence in *A. thaliana*, whereas the appearance of senescence symptoms is delayed in plants lacking functional ORS1. Furthermore, 42 genes were shown to be induced by ORS1, many of which are known to be involved in age-dependent senescence and in the response to long-term salinity (Balazadeh et al., 2011). Of the WRKY transcription factors, *WRKY53* is one of the most studied genes with regard to senescence. It can affect the expression of several other transcription factors including other WRKYs, indicating that it might be a key player in a transcription factor signaling cascade (Miao et al., 2004). In addition, the MAP3K MEKK1 can directly phosphorylate the WRKY53 protein thereby increasing its DNA-binding activity, suggesting that MAPK signaling is also involved in the regulation of senescence (Miao et al., 2007). This idea is supported by the fact that plants overexpressing or lacking MKK9 and MPK6 show an accelerated or delayed onset of senescence, respectively (Zhou et al., 2009).

As mentioned above, metal exposure induces many effects associated with senescence in a broad range of plant species (**Table 2**). McCarthy et al. (2001) demonstrated Cd-induced increases in lipid peroxidation and protease activity in *P. sativum* leaves. Furthermore, they reported a decreased leaf chlorophyll content and a disorganization of chloroplast structure in leaves of Cd-exposed plants. Similar results were obtained by Rodríguez-Serrano et al. (2006), showing Cd-induced lipid peroxidation in *P. sativum* roots. In addition, levels of the senescence-promoting phytohormones SA, JA and ethylene were significantly elevated in roots of Cd-exposed plants as compared to those of control plants. Interestingly, these changes were accompanied by increases in $O_2^{\bullet-}$ and H_2O_2 levels, suggesting a role for ROS in Cd-induced accelerated senescence. In addition to Cd, other metals were shown to induce senescence-associated processes as well. Upadhyay and Panda (2010) demonstrated lipid peroxidation and decreased chlorophyll content associated with increased ROS levels in *Spirodela polyrhiza*. Furthermore, lipid peroxidation and negative effects on chlorophyll content or chloroplast structure were reported in Pb-exposed *Ceratophyllum demersum* (Mishra et al., 2006) and Zn-exposed *Hydrilla verticillata* (Xu et al., 2013).

Taken together, these data strongly suggest that metal exposure induces accelerated senescence in plants. However, little or no data are available on the effect of metal exposure on SAG expression levels. It is known, however, that transcription of many SAGs is increased in plants treated with H_2O_2 (Miao et al., 2004; Yan et al., 2007; Zhou et al., 2013, Zhou et al., 2014). Interestingly, *ORS1* and *WRKY53* expression was also induced by H_2O_2, suggesting that both transcription factors play a key role in the H_2O_2-induced senescence response in plants (Miao et al., 2004; Balazadeh et al., 2011).

A role for ROS in regulating senescence is further supported by the observed increased concentrations of $O_2^{\bullet-}$ and H_2O_2 in senescing tissues (Fischer, 2012). This can be caused by lipid peroxidation, which is known to occur during senescence (Zimmermann and Zentgraf, 2005). However, it could also be due to a decrease in the plant's antioxidative defense as reported by several authors (Jiménez et al., 1998; Prochazkova et al., 2001; Procházková and Wilhelmová, 2007). This hypothesis is further supported by the fact that the *Arabidopsis vtc1-1* mutant, which is deficient in the antioxidative metabolite AsA, has a higher expression of certain SAGs and an earlier appearance of senescence symptoms as compared to WT plants (Barth et al., 2004). In addition to AsA, also the antioxidative enzyme CAT could be involved in regulating senescence. Indeed, Zimmermann et al. (2006) proposed that a downregulation of the *CAT2* isoform contributes to the senescence-associated H_2O_2 peak, subsequently causing an increase in the expression levels of the stress-responsive *CAT3* gene. Interestingly, Cuypers et al. (2011) reported a downregulation of *CAT2* and an upregulation of *CAT3* in Cd-exposed *A. thaliana* plants, possibly pointing to a Cd-induced acceleration of senescence.

As metals are known to increase ROS production, thereby inducing an oxidative challenge, we hypothesize a role for H_2O_2 in the damage and signaling events ultimately leading to premature leaf senescence under metal stress. In order to gain more insight into the effect of metal exposure on leaf senescence, future research should aim to identify the influence of different metals on the expression levels of SAGs including transcription factors such as ORS1 and WRKY53.

CONCLUSIONS AND A LOOK FORWARD

By compiling the gathered evidence, the role of ROS and particularly H_2O_2 in regulating metal stress responses in plants is unequivocally demonstrated. Furthermore, it is becoming increasingly clear that oxidative damage and signaling are two sides of the same coin, potentially cooperating to establish plant acclimation and tolerance to metal exposure. Different studies highlight the interaction between ROS/H_2O_2 and signaling components such as MAPKs, phytohormones, Ca^{2+}, $NO^{\bullet}$, oxylipins and regulating systems like miRNAs (**Figure 2**). Nevertheless, our current knowledge only represents the tip of the iceberg, encouraging further research efforts in the field of H_2O_2 perception, signal transduction and its role in plant acclimation to and growth under metal stress conditions.

AUTHOR CONTRIBUTIONS

All authors participated in the conception of the topic. AC, SH and EK wrote the manuscript. Figures and Tables were designed by RAR, SDS, JD, HG, MJ, CL and HV. All authors read and approved the final manuscript after critically revising it for important intellectual content.

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Cross Talk between H_2O_2 and Interacting Signal Molecules under Plant Stress Response

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It is well established that oxidative stress is an important cause of cellular damage. During stress conditions, plants have evolved regulatory mechanisms to adapt to various environmental stresses. One of the consequences of stress is an increase in the cellular concentration of reactive oxygen species, which is subsequently converted to H_2O_2. H_2O_2 is continuously produced as the byproduct of oxidative plant aerobic metabolism. Organelles with a high oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria, or peroxisomes are major sources of H_2O_2 production. H_2O_2 acts as a versatile molecule because of its dual role in cells. Under normal conditions, H_2O_2 immerges as an important factor during many biological processes. It has been established that it acts as a secondary messenger in signal transduction networks. In this review, we discuss potential roles of H_2O_2 and other signaling molecules during various stress responses.

Keywords: H_2O_2, ROS, abscisic acid, nitric oxide, biotic/abiotic stress, phytohormones

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INTRODUCTION

In plants, reactive oxygen species (ROS) are continuously produced in different cellular compartments as byproducts of various metabolic pathways such as respiration and photosynthesis. All aerobic organisms use molecular oxygen as terminal oxidant during respiration. Oxygen is neither very reactive nor harmful, but it has the potential to be only partially reduced, leading to the formation of very reactive and therefore toxic intermediates like singlet oxygen ($_1O_2$), superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH). All ROS are extremely reactive, causing damage to membranes and other cellular components. ROS also have strong affinities toward membrane, DNA, proteins, carbohydrates and lipids in plant cells (Anjum et al., 2015; Jajic et al., 2015). Hence, these molecules are constantly scavenged by different non-enzymatic and enzymatic detoxification mechanisms that are often confined to particular compartments (Alscher et al., 1997). It is important to remove ROS from the plant system in order to abate stress response, taking also into account that the final consequence of an eventual disequilibrium due to adverse environmental factors is the rapid increase of intracellular ROS levels, the so-called "oxidative burst" (Sharma et al., 2012). However, the balance between frequent production and scavenging of ROS may be disturbed by a number of adverse environmental factors such as light, temperature, drought, salinity, cold, heavy metal ions, UV exposure and water (Boyer, 1982). The usual external stress factors that affect ROS production in plants can be biotic (executed by other organisms) or abiotic (arising from changes in chemical of physical environment). However, in plants the constant regulation of the ROS concentration,

including H_2O_2, is controlled by the enzymes such as catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione *S*-transferases (GSTs), glutathione reductase (GR), and peroxyredoxin; and non-enzymatic compounds, like ascorbate, glutathione (GSH), α-tocopherol and flavonoids (Kapoor et al., 2015).

Recent studies have elucidated that under different stress conditions, plants react in a very complex manner which includes various physiological and cellular changes (Atkinson and Urwin, 2012). In order to combat stress response, plants use various signaling mechanisms derived from hormonal regulations. Nevertheless, recent studies indicate that plants also make use of ROS as signaling molecules for regulating development and various physiological responses. There is also increasing focus on ROS production and its integration with various hormonal signaling pathway in regulation of plant growth and stress tolerance (Xia et al., 2015).

Amongst all, superoxide and H_2O_2 are two ROS that have been given more importance in recent studies. The main focus of this review is on H_2O_2. H_2O_2 is freely diffusible across membranes, which enables it to diffuse the damage. It is relatively long lived and it acts as a central player in stress signal transduction pathways (Møller et al., 2007). Thenard was the first in 1818 to isolate H_2O_2 which later came across as a cell damaging molecule when accumulated at higher concentrations in the cell (Plaine, 1955). In early 90 s Ievinsh and Tiberg also predicted the role of H_2O_2 as a signaling molecule (Ievinsh and Tiberg, 1995). Based on earlier studies, it is certain that H_2O_2 is part of oxidative metabolism and is involved in various metabolism and signaling cascades which are essential for plant growth and development, such as development of root hair, reinforcement of plant cell wall, xylem differentiation, resistance enhancement, cell wall structural cross linking and cell wall loosening in stomatal control (Dempsey and Klessig, 1995).

H_2O_2 being a versatile molecule acts as an important signal at normal levels, whereas under abiotic or biotic stress conditions it induces oxidative stress. Its unique property of stability and less reactivity differentiates H_2O_2 from the rest of the ROS molecules (Yang and Poovaiah, 2002; Quan et al., 2008), thus making it a good signaling molecule. In plants, H_2O_2 works as a key factor in non-toxic level concentration. As a signaling molecule, it shows tolerance to biotic and abiotic stress by getting involved in various pathways (Mittler et al., 2004; Reczek and Chandel, 2015). At toxic concentration levels H_2O_2 showed involvement in programmed cell death (PCD; Dat et al., 2003). In a recent article, the dual nature of H_2O_2 has been studied where 600 μM H_2O_2 treatment caused an increase in the vase life of hybrid lily "Manissa," while an increase in concentrations resulted in negative effects (Liao et al., 2012).

Several studies have indicated that H_2O_2 interplays with other signaling molecules such as abscisic acid (ABA) and ethylene which are important for plant development and senescence (Jubany-Mari et al., 2009; Chen et al., 2012). **Table 1** shows recent studies unveiling the mechanism by which H_2O_2 is involved in various biological processes. A recent study indicated the involvement of nitric oxide (NO) and H_2O_2 in salicylic acid (SA)-induced salvianolic acid B production in *Salvia miltiorrhiza* cell culture. Increase in H_2O_2 production has been observed by SA despite being inhibited by IMD (an inhibitor of NADPH oxidase) or DMTU (a quencher of H_2O_2) which further increases NO production and Sal B accumulation (Guo et al., 2014). In mung bean seedlings SA also plays roles in adventitious root formation and its effect on H_2O_2 accumulation has been observed. It has been concluded from the study that H_2O_2 accumulation acts as a downstream process in regulation with SA induced adventitious root formation (Yang et al., 2013).

H_2O_2 pretreatment in maize leaves significantly increased ABA content (Terzi et al., 2014). Pre-treatment of sodium nitroprusside (SNP) and SA in wheat seedlings decreased the effect of osmotic stress. It was also observed that pre-treatment of seedlings with methylene blue (a guanylatecyclase inhibitor) diminished the protective effects of both SA and SNP. Therefore, it is concluded that protective effect may only be limited to NO (Alavi et al., 2014). Another study on jasmonate revealed that 12-oxo phytodienoic acid is involved in reduced H_2O_2 accumulation (Taki-Nakano et al., 2014). Yang (2014), proposed a model describing three pathways in modulating the transcription factor *AtERF73/HRE1* which includes ethylene dependent, ethylene-independent/H_2O_2-dependent pathway, and an ethylene and H_2O_2-independent pathway. This study also proposes involvement of H_2O_2 and ethylene in *AtERF73/HRE1*and *ADH1* gene expression under stress. There is another study stating the involvement of ethylene in H_2O_2 accumulation during PCD (de Jong et al., 2002). In drought stress, calcium-dependent protein kinase (*CPK8*) has been involved in ABA-mediated stomatal regulation via *CAT3* (*CPK8*-interacting protein) activity. It also has been observed that *cpk8* and *cat3* plants showed reduced CAT activity and higher H_2O_2 accumulation (Zou et al., 2015).

Guo et al. (2014) studied the possibility of SA involvement in H_2O_2 and NO induced salvianolic acid B accumulation; where the main function of NO is to downstream SA signaling which results in reduced oxidative stress (Alavi et al., 2014). Exogenous application of H_2O_2 and its induction in high light showed different effects on gene expression (Olemiec et al., 2014). It was shown that H_2O_2 could be involved in the signaling of plant growth regulators such as ethephon (Chen et al., 2012). The application of ethephon results in an elevation in H_2O_2 levels, which is accompanied by the increased expression of sweet potato CAT.

H_2O_2 PRODUCTION IN PLANT CELLS AND ITS REACTIVITY IN DIFFERENT CELLULAR COMPARTMENTS

H_2O_2 is produced in photosynthesizing cells, majorly in peroxisomes (photosynthetic carbon oxidation cycle) and minorly in choroloplast, mitochondria (respiratory electron transport chain) (**Figure 1**), the endoplasmic reticulum (ER), nucleus and plasma membranes (del Río et al., 2006; Bhattacharjee, 2012). Peroxisomes and chloroplasts may accumulate 30–100 times higher H_2O_2 as compared to mitochondria (Hossain et al., 2015). H_2O_2 production occurs

TABLE 1 | Recent studies showing the interrelation between H_2O_2 and its interaction with signaling molecules.

Hormone and interacting molecule	Plant	Interaction with H_2O_2	Reference
Nitric oxide	*Salvia miltiorrhiza*	Elicitation of SA for either H_2O_2 or NO was independent, and the elicited H_2O_2 or NO could act independently or synergistically to induce Sal B accumulation in SA-elicited cells.	Guo et al., 2014
Salicylic acid	Mung bean	Pretreatment of mung bean explants with N, *N*′-dimethylthiourea (DMTU), a scavenger for H_2O_2, resulted in a significant reduction of SA-induced ARF.	Yang et al., 2013
Ethylene	Tomato	Ethylene is a potentiator of the camptothecin-induced oxidative burst and subsequent PCD in tomato cells.	de Jong et al., 2002
Abscisic acid	Maize	H_2O_2 pretreatment may alleviate water loss and induce osmotic stress resistance by increasing the levels of soluble sugars, proline, and polyamines thus ABA and H_2O_2 production slightly decrease in maize seedlings under osmotic stress.	Terzi et al., 2014
Salicylic acid and nitric oxide	Wheat	SA and NO pretreatment reduces H_2O_2 level in wheat	Alavi et al., 2014
Jasmonate	Not applicable	Among the jasmonates, only 12-oxo phytodienoic acid (OPDA) suppressed H_2O_2-induced cytotoxicity. OPDA pretreatment also inhibited the H_2O_2-induced ROS increase and mitochondrial membrane potential decrease.	Taki-Nakano et al., 2014
Ethylene	*Arabidopsis*	H_2O_2 and ethylene interplay has an effect on AtERF73/HRE1 and ADH1 expression during the early stages of hypoxia signaling.	Yang, 2014
Abscisic acid and Calcium	*Arabidopsis*	ABA, H_2O_2, and Ca^{2+}-induced stomatal closing were impaired in *Arabidopsis*.	Zou et al., 2015

during the oxidative burst when reduction of molecular oxygen (O_2) into superoxide anion ($O_2^{\bullet -}$) occurs (Sutherland, 1991). At the cell wall this $O_2^{\bullet -}$ undergoes spontaneous dismutation at a higher rate and at an acidic pH. Nicotinamide adenine dinucleotide (NADH) undergoes oxidation to form NAD^+ by cell wall peroxidase with further reduction of O_2 to $O_2^{\bullet -}$ resulting in the production of O_2 and H_2O_2 (Bhattacharjee, 2005). In apoplast, amine oxidase and germin-like oxidase have been proposed to generate H_2O_2 (Bolwell and Wojtaszek, 1997). Cell membrane NADPH-dependent oxidase serves as a H_2O_2 source. H_2O_2 production occurs in the cell via reaction between the oxygen molecules (O_2), forming superoxide anion ($O_2^{\bullet -}$). During the stress response O_2 is reduced to $O_2^{\bullet -}$ which undergoes spontaneous dismutation (Sutherland, 1991). $O_2^{\bullet -}$ is also catalyzed and reduced by superoxide dismutase (SOD) and protein kinase C (PKC) to form H_2O_2 (Scandalios, 1993). SOD enzyme catalyzes $O_2^{\bullet -}$ which mainly occurs in cytosol, chloroplast and mitochondria (Scandalios, 1993). Rather than superoxide disproportionation, H_2O_2 is also produced by $O_2^{\bullet -}$ reduction by reductants such as ferredoxins, thiols, ascorbate (Asada and Takahashi, 1987). PKC also shows involvement in H_2O_2 production. OH is produced in the reaction of H_2O_2 with Fe^{2+} (Arora et al., 2002). To maintain the balance between H_2O_2-scavenging enzymes and SODs, equilibrium for H_2O_2 concentration in cells should be attained (Mittler et al., 2004).

H_2O_2 production in chloroplasts originates from several locations and occurs mainly in Chl associated with the photosynthetic electron transport chain (PET) which is the primary source of O_2. In chloroplast, molecular oxygen (O_2) uptake is associated with photoreduction of O_2 to superoxide radical ($O_2^{\bullet -}$). Singlet oxygen ($_1O_2$) is produced by energy transfer to triplet oxygen ($_3O_2$) in photosystem II (PSII). Photosystem II excitation results in the oxidation of water (H_2O) to O_2 (**Figure 1A**). The reductant formed by this process donates electrons (e^-) to plastoquinone (PQ). These e^- eventually passes through the cytochrome *f* (Cyt *f*) complex, plastocyanin (PC), and then to photosystem I (PSI). The PET chain includes a number of enzymes on the reducing (acceptor) side of photo system I (PSI): Fe–S centers, reduced thioredoxin (TRX), and ferredoxin. When ferredoxin (Fd) is over reduced during photosynthesis electron transfer, electron may be transferred from photosystem I (PSI) to O_2 to form $O_2^{\bullet -}$ by the process called Mehler reaction. The $O_2^{\bullet -}$ formed then undergoes dismutation to hydrogen peroxide (H_2O_2) either spontaneously or facilitated by SOD (**Figure 1A**).

In plants, the main function of peroxisome is photorespiration which involves O_2 uptake (light-mediated) and the emission of CO_2 in simulation with H_2O_2 formation (Dat et al., 2000). H_2O_2 production in peroxisome results from the oxygenase activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Noctor et al., 1999). It is being stated that plants exposed to favorable conditions for oxygenation are more subjected to produce H_2O_2 (Foyer and Noctor, 2000). H_2O_2 is generated during the oxidation of glycolate in the C2 cycle of peroxisomes (**Figure 1B**). Production of H_2O_2 is attributed to the matrix-localized enzyme, xanthine oxidase (XOD), which catalyses the oxidation of xanthine or hypoxanthine to uric acid, and

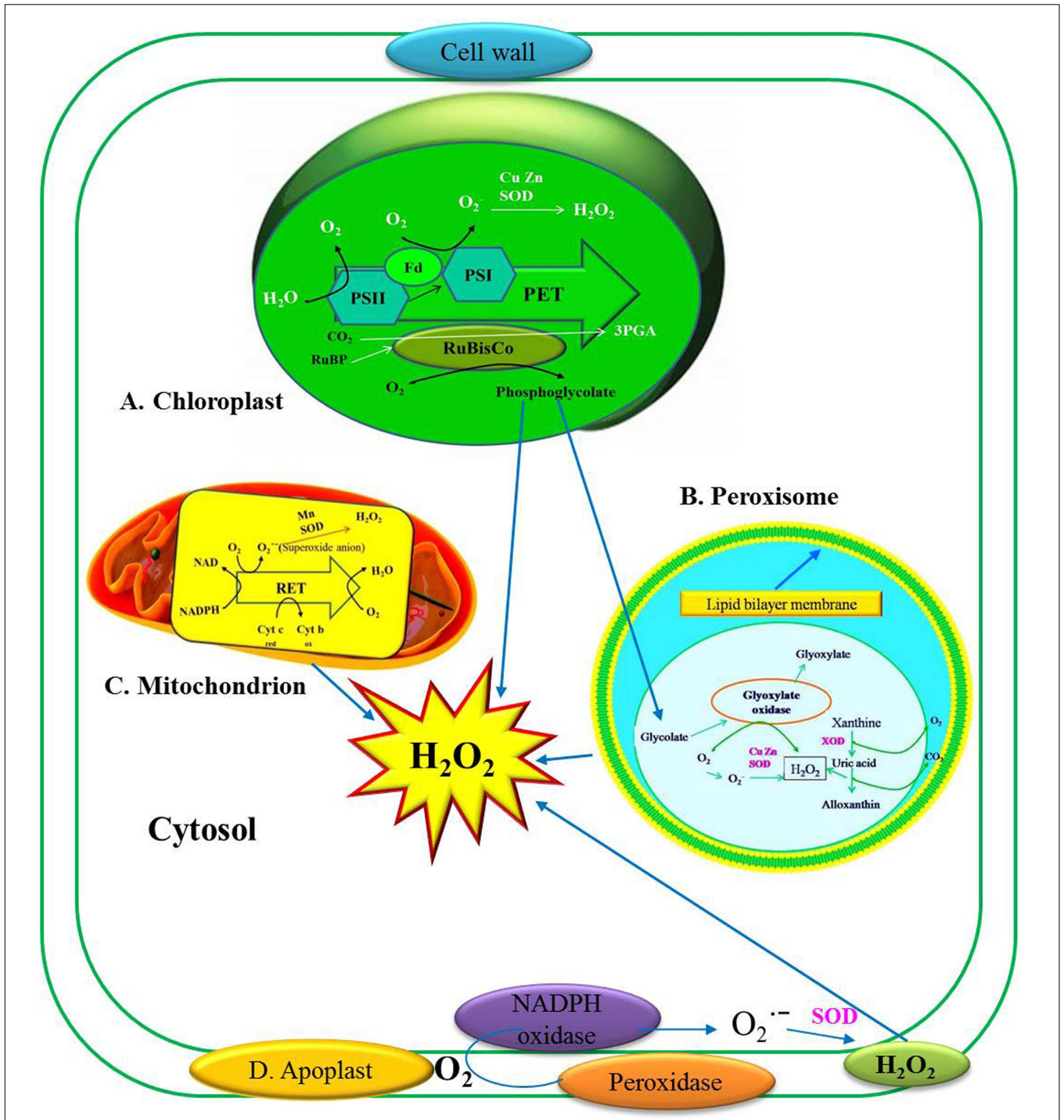

FIGURE 1 | Production of hydrogen peroxide (H_2O_2) in plant cells and their reactivity in various cellular components. (A) H_2O_2 production in Chloroplast at the site of PSI and PSII. **(B)** H_2O_2 production in Peroxisomes. XOD (Xanthine oxidase), GOX (glycolate oxidase). **(C)** H_2O_2 production in Mitochondria. **(D)** H_2O_2 production in Apoplast (Figure modified from Jajic et al., 2015).

is a well-known producer of $O_2^{\bullet-}$ in the process. This $O_2^{\bullet-}$ is then converted into H_2O_2 by Cu, Zn-SOD (**Figure 1B**) (Corpas et al., 2001). An NAD(P)H-dependent $O_2^{\bullet-}$ production site which uses $O_2^{\bullet-}$ as an electron acceptor is present in the peroxisomal membrane and it releases the $O_2^{\bullet-}$ into the cytosol. This site appears to be formed by a small ETC which is composed of a flavoprotein NADH and Cyt *b* (del Río et al., 2006).

Two major mitochondrial sites for superoxide radical production in electron transport chain are cytochrome bc1 complex and NAD(P)H dehydrogenases (Moller, 2001). The mitochondrial respiratory electron transport (RET) harbors electrons with sufficient free energy to directly reduce O_2, which is considered as a primary source of H_2O_2 generation (**Figure 1C**). This direct reduction of O_2 to $O_2^{\bullet -}$ takes place in the flavoprotein region of the NADH dehydrogenase segment, which is responsible for the O_2 consumption. During RET, the oxygen radical is markedly enhanced in the presence of antimycin A, which blocks the electron flow after ubiquinone (UQ). Autooxidation of reduced UQ results in the production of $O_2^{\bullet -}$ which is a major precursor of H_2O_2 production in UQ location of the RET. Completely reduced UQ donates e^- to cytochrome c (Cyt *c*) and leaves an unstable, highly reducing semiquinone species, which would normally reduce cytochrome *b* (Cyt *b*), which instead reduces the O_2 to $O_2^{\bullet -}$ (**Figure 1C**). This $O_2^{\bullet -}$ is further reduced by manganese SOD (Mn-SOD) dismutation to H_2O_2 (Moller, 2001).

Some other sources of H_2O_2 production are plasma membrane, cytoplasm and the extracellular matrix (ECM). There are various enzymes responsible for H_2O_2 production in plasma membrane (Vianello and Macri, 1991). H_2O_2 production in cytoplasm occurs by the electron transport chain which is associated with the ER. This oxidation and hydroxylation process involve cytochrome P450 and cytochrome P450 reductase whereas, fatty acid desaturation involves cytochrome b_5 and cytochrome b_5 reductase, which donate electrons for H_2O_2 formation (Bartosz, 1997; Mittler et al., 2004).

Studies have shown that NADPH oxidase at the plasma membrane in the plant cell is involved in plant defense reactions to pathogen attack (Torres et al., 2002) and in response to various abiotic stresses (Kwak et al., 2003). The NADPH-dependent oxidase system sometimes referred to as *rboh* (for respiratory burst oxidase homolog), similar to that present in mammalian neutrophils, has received the most attention. It catalyzes the production of $O_2^{\bullet -}$ by one-electron reduction of oxygen using NADPH as the electron donor (Desikan et al., 2003; Mahalingam and Federoff, 2003; Apel and Hirt, 2004). The superoxide anion radical is most likely located in the apoplastic space and is converted to H_2O_2 either spontaneously or by extracellular SOD (Karpinska et al., 2001; Bolwell et al., 2002) (**Figure 1D**). There are other plant species which NADPH oxidase or *rboh* genes have been cloned (Desikan et al., 2003).

ABSCISIC ACID AND INTERACTION WITH H_2O_2

Abscisic acid is one of the crucial phytohormones which play important roles under various environmental cues. It accumulates as a response to stressful environmental conditions such as dehydration, cold temperature or shortened day length. The application of ABA plays fundamental role in different physiological processes and biological pathways such as seed dormancy and delay in germination, development of seeds, promotion of stomatal closure, embryo morphogenesis, synthesis of storage proteins and lipids, leaf senescence and also defense against pathogen (Swamy and Smith, 1999).

It has been reported that the ABA signaling pathway is identified as a central regulator of abiotic stress response in plants, triggering major changes in gene expression and adaptive physiological responses (**Figure 2**). Recently, MAPK (mitogen activated protein kinase) cascades have also been shown to be implicated in ABA signaling. External ABA treated plants induced the transcriptional regulations, protein accumulation and stability, and kinase activity of several components of distinct MAPK signaling cascades in many plant species. These existing evidences suggest that MAPK cascades are actively involved in several ABA responses, including abiotic stress defense mechanisms and guard cell signaling (Zhang et al., 2007; Jammes et al., 2009; Zong et al., 2009). Rodriguez et al. (2010) reported that the MAPK cascade is activated by the exogenous H_2O_2 which in turn is mediated by the hormones like ABA, Jasmonic acid (JA) and SA.

In plants, MAPK cascades are generally organized pathways in which the upstream activated MAPK kinase kinase (MAPKKK) undergoes the sequential phosphorylation and subsequent activation of downstream MAPK kinases (MAPKKs) and MAPKs. This MAPK cascades translate incoming environmental signals into post-translational modifications of target proteins that ultimately reorganize gene expression and stress adaptation. In *Arabidopsis,* H_2O_2 activates the MAPKs (MPK3 and MPK6) via the MAPK kinase kinase (MAPKKK) ANP1 (Kovtun et al., 2000). MKP2 is a key regulator of the MPK3 and MPK6 networks that are involved in controlling both abiotic and biotic stress responses (Zhou et al. (2012). Treatment with ABA in *Arabidopsis* plants induced the transcriptional regulation of MPK3, MPK5, MPK7, MPK18, MPK20, MKK9, MAPKKK1 (ANP1), MAPKKK10 (MEKK3), MAPKKK14, MAPKKK15, MAPKKK16, MAPKKK17, MAPKKK18, MAPKKK19, *Raf6*, *Raf12,* and *Raf35* (Menges et al., 2008; Wang et al., 2011) signifying a promising role in ABA signaling. Recently, Jammes et al. (2009) used a cell type-specific functional genomics approach and identified that the MAPKs, MPK9 and MPK12 were preferentially expressed in guard cells. It was also reported that MPK12 were activated by ABA and H_2O_2. MPK9 and MPK12 mediate ABA signals in guard cells (Montillet et al., 2013). In *Arabidopsis*, a T-DNA *oxi1* null mutant is impaired in the activation of the two MAPKs, MPK3 and MPK6, upon oxidative stress (Rentel et al., 2004), suggesting that serine/threonine kinase OXI1 functions downstream of ROS but upstream of the MAPK module. H_2O_2 also increases the expression of the *Arabidopsis* nucleoside diphosphate (NDP) kinase 2 (Moon et al., 2003). H_2O_2 accumulation was reduced by the over expression of At-NDPK2 and enhanced tolerance to multiple stresses, including cold, salt, and oxidative stress. Moreover, the MAPKs, MPK3 and MPK6 are activated by H_2O_2 induced NDPK2. Gechev et al. (2005) identified a transcription factor ANAC42 and reported that it was highly regulated by H_2O_2 which in turn is achieved through OXI1/MPK3 and MPK6 activation (**Figure 2**). Interestingly, ANAC42 plays a role in senescence and H_2O_2 induced PCD

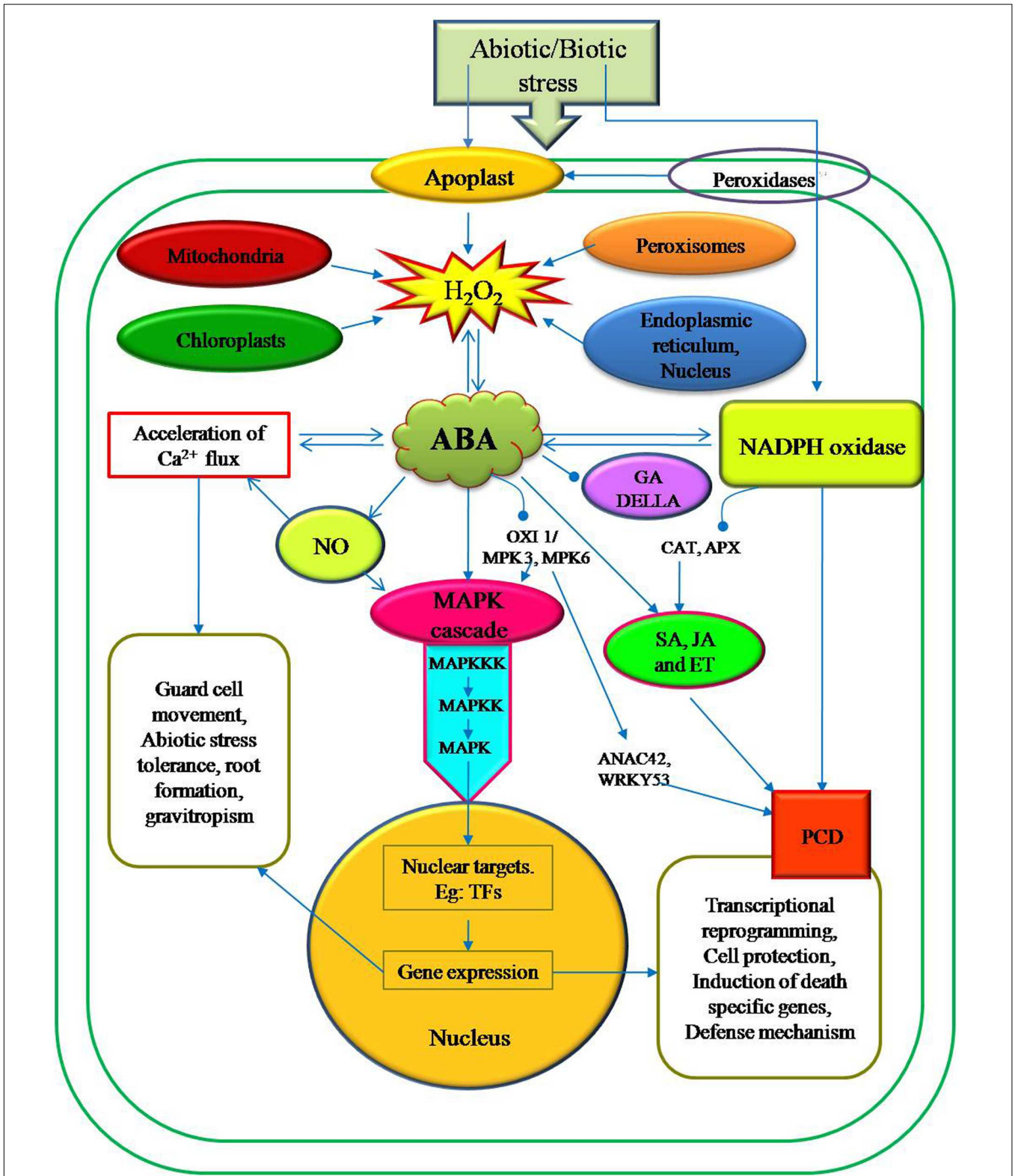

FIGURE 2 | Schematic representation of H_2O_2 generation in various intra-and extra cellular sites and its involvement in the various signaling pathways associated with the regulation of defense gene expression in plant cells. ABA is extensively associated with wide range of abiotic stress signals and administers with growth and development processes in plants. In contrast to ABA, other phytohormones such as SA, GA, JA, and ethylene have significant role during biotic stress tolerance in plants. Nevertheless, ABA is an important signaling molecule in H_2O_2 production and activating the MAPK cascade by interacting with other plant hormones.

(Fujii, 2014). Also, the MAPKKK can interact directly with WRKY53, a transcription factor that involved in senescence induced PCD (**Figure 2**), thus bypassing the downstream kinases (Miao et al., 2006). Nevertheless, it is clear from the past reports that MPK3 and MPK6 are two important integrating points of signals from cellular developmental programs and the external environment changes (Wang et al., 2008). Even though most of the connections between ABA and MAPKs are poorly understood, it is evident that these pathways are part of the complex cellular signaling network of plants to integrate various environmental signals (Danquah et al., 2014).

One of the most important functions of ABA is to induce stomatal closure by reducing the turgor of guard cells under water deficit. There is evidence that H_2O_2 may function as an intermediate in ABA signaling in *Vicia faba* guard cells (Pei et al., 2000). There are reports which suggest that H_2O_2 is one of the major ROS and plays an important role as a second messenger in ABA induced stomatal closure (Pei et al., 2000; Miao et al., 2006). While ABA induced the synthesis of H_2O_2 and superoxide under stressful condition and caused oxidative stress (Guan et al., 2000). However, ABA is a natural growth regulator which accumulates in plants under plant stress conditions (Zheng et al., 2010). Results of (Zhang et al., 2001) provide evidence for H_2O_2 involvement in ABA induced stomatal movement in *V. faba.* Major findings of this study showed stomatal closure by exogenous application of H_2O_2 with ABA whereas, reverse action of H_2O_2 generation by scavenging its activity results in reduced stomatal closure (Guan et al., 2000). Overproduction of ABA induces H_2O_2 accumulation which enhances the stomatal closure by activating plasma membrane calcium channels (Pei et al., 2000). Plasma membrane-located NADPH oxidases Atrboh D and Atrboh F were found to be required for ABA-induced stomatal ROS production (Kwak et al., 2003). It has been reported that during oxidative burst, NADPH oxidase can trigger Ca^{2+}, and mitogen-activated protein kinase (MAPK) signaling pathways as well as suppress the hormone signal transfer routes such as SA, JA, and ethylene (Overmyer et al., 2003; Evans et al., 2005) (**Figure 2**). Abiotic stress condition enhances ABA/gibberellic acid (GA) ratio supporting DELLA protein accumulation which in turn lowers H_2O_2 levels (Considine and Foyer, 2014). In rice seeds, ABA decreased ROS production, which leads to a repression of ascorbate and GA accumulation (Ye et al., 2012). It was also reported that H_2O_2 is involved in GA/ABA signaling in barley aleurone cells (Ishibashi et al., 2012). Calcium and calmodulin (CaM)-dependent protein kinase (CCaMK) belongs to calcium/CaM-dependent protein kinase superfamily (Harmon et al., 2000); activation by free Ca^{2+} and Ca^{2+} bound to CaM has been indicated to be involved in ABA signaling during abiotic stress responses (Yang et al., 2011) and ABA-induced antioxidant defense by functioning upstream of ABA-activated MAPK (Shi et al., 2014).

These available pieces of evidence clearly indicate that the ABA is a key hormone in inducing abiotic stress responses. Downstream events mediated by MAPK cascade, alterations in Ca^{2+} fluxes and the activation of ion channels changes the redox state of the cell. All these actions lead to transcriptional reprogramming, which results in target gene expression such as cell death specific nucleases and proteases, and eventually PCD (**Figure 2**). Most of the genes (NADPH oxidases, SOD and extracellular peroxidases) expressed in the early signals are involved in the ROS generation, essential for triggering PCD. While other genes are responsible for maintaining ROS levels (CAT and APXs) (Gadjev et al., 2008) (**Figure 2**).

CALCIUM AND INTERACTION WITH H_2O_2

Calcium (Ca^{2+}) is important for robust cell wall formation. It is also crucial for the root system and in young, growing cells. Alteration in Ca^{2+} level is observed under shifting environmental conditions, including changes in light, temperature, and drought (Mahajan and Tuteja, 2005). Ca^{2+} is significant for cross-linking acidic pectin residues and permeability of the plasma membrane. As a secondary messenger, Ca^{2+} concentration is balanced by storing it in vacuoles and is released whenever necessary (Mahajan and Tuteja, 2005). It is indispensable for all important signaling pathways. Plant signaling network has the capability to alter its functioning under various environmental challenges. The plant cell primary response under stress condition is a modification in plasma membrane permeability leading to calcium and proton influx that appears to be necessary and sufficient for the induction of H_2O_2 (Pei et al., 2000). Plant metabolism involves various Ca^{2+}/CaM proteins having different functions out of which some are involved in H_2O_2 signaling such as NAD kinase (Harding et al., 1997). CAT is also important for H_2O_2 regulation and its deficiency can lead to H_2O_2 accumulation (Gechev et al., 2004). H_2O_2 and Ca^{2+} interrelation study has been shown by Yang and Poovaiah (2002) in *Arabidopsis*. Another study on *Arabidopsis* seedlings suggests the role of H_2O_2 in biphasic Ca^{2+} elevation, with the first peak located in cotyledons and the second in the root (Rentel and Knight, 2004). The antioxidant system may also be considered as a target of Ca^{2+} influence, for example, the efficiency of H_2O_2 scavenging in *Arabidopsis* plants depends on the peroxisomal Ca^{2+} concentration (Costa et al., 2010).

Continuous Ca^{2+} invasion is most importantly required for H_2O_2 accumulation which also activates NADPH oxidase located in the plasma membrane (Lamb and Dixon, 1997). There is another study suggesting the role of biphasic (Ca^{2+}) and H_2O_2 in aequorin tobacco cell culture's expression (Lecourieux et al., 2002). Pollen tube growth has been enhanced by H_2O_2 regulated spermidine oxidase, which also induces Ca^{2+} channel (Wu et al., 2010). H_2O_2 involvements in Ca^{2+} influx via Ca^{2+} permeable channel and partial stomatal closure were observed in the study (Pei et al., 2000). Significant induction in nuclear and cytosolic Ca^{2+} level by free sphingoid Long Chain Base (LCB) sphinganine has been observed in simulation with decreased accumulation of H_2O_2 in tobacco cells (Lachaud et al., 2011). Later studies have revealed that CAT can scavenge

H_2O_2 production which is likely mediated by Ca^{2+} homeostasis in *Arabidopsis* (Suzuki et al., 2011). In this case, cytoplasmic Ca^{2+} was shown to bind to *rboh* at the N-terminal region and thus to promote the activation of *rboh* and produce H_2O_2 (Takeda et al., 2008). H_2O_2 mediated rapid gene expression (*LeCDPK1*) in tomato leaves has been observed (Chico et al., 2002) whereas, H_2O_2 treatment in wheat plant also leads to enhanced responsiveness in eight out of 20 studied calcium-dependent protein kinase (CDPKs) (Li et al., 2008). Induction in gene expression (*GST1*) by Ca^{2+}response in association with H_2O_2 may be due to changes in redox balance (Rentel and Knight, 2004).

NITRIC OXIDE AND INTERACTION WITH H_2O_2

Increasing evidence based on experiments has shown a vital role of NO in protecting plants against stress conditions (Wink et al., 1993). It is generated in higher plants through oxidative (arginine or hydroxylamine-dependent) and reductive (nitrate-dependent) pathways (Gupta et al., 2011). NO being part of various physiological processes in plant system makes it one of the major signaling molecules. Initially NO was considered to be a toxic gas. However, this idea changed after the discovery of the signaling role of NO in regulating the cardiovascular system (Skovgaarda et al., 2005). One of the major areas in the study of NO is its involvement in coordinating several defense responses during both biotic and abiotic stress conditions in the plants. In the past 2 decades, much focus was given to NO related studies toward its role in plant defense system under oxidative stress. Studies on adaptive mechanisms of plants have shown an increased basal level of NO in water and heat stressed plants, suggesting its importance in abating stress (Leshem and Haramaty, 1996; Leshem et al., 1998). The defensive mechanism of NO in plants under oxidative stress is associated with its ability to function as an antioxidant by directly scavenging the ROS, thus reducing cellular damage (Romero-puertas and Sandalio, 2016) and acting as a signaling molecule which eventually results in changes in gene expression (Lamattina et al., 2003).

A study focused on H_2O_2 generation in simultaneous correlation with NO production was shown during the hypersensitive response (HR) in which both cooperates to trigger hypersensitive cell death (Delledonne et al., 2001). The function of NO is tightly linked to ROS and it has a half-life of only a few seconds, once produced, interacts rapidly with ROS, giving rise to a number of reactive nitrogen species, such as nitrogen dioxide (NO_2), which degrades to nitrite and nitrate in aqueous solutions (Neill et al., 2008; Bellin et al., 2013). There were studies showing involvement of both NO and H_2O_2 in bacterially induced PCD in soybean where both signals work synergistically (Delledonne et al., 1998) and in *Arabidopsis* they work as additive (Clarke et al., 2000). H_2O_2 formation may occur via superoxide radical ($O_2^{\bullet -}$). There is a probability that NO reacts with $O_2^{\bullet -}$ to form highly reactive peroxynitrite anion ONOO- and subsequent cellular effects may then be induced by peroxynitrite (Bellin et al., 2013).

In mammals, NO has been shown to react with glutathione to form *S*-nitrosoglutathione (GSNO) which serves as a systemic source of NO and a similar situation has been suggested for plants (Durner and Klessig, 1999). It is clear that both H_2O_2 and NO can mediate transcription, but the involved processes remain unclear. There is a possibility of both H_2O_2 and NO having a direct effect on transcription factors by *S*-nitrosylation and oxidation of cysteine. A recent study suggests characterization of redox-sensitive factor in yeast where H_2O_2 oxidation alters the activity of this protein (Delaunay et al., 2000). Phosphorylation of cascade such as the mitogen-activated protein kinases (MAPK) is suggested to play another important role on H_2O_2 and NO. There is another study on tobacco Bright Yellow-2 (TBY-2) cells, suggesting an involvement of both H_2O_2 and NO in the activation of PCD, and treatment of scavenger for both the signaling molecules restores the cell viability (De Pinto et al., 2006).

In a new study, the cloning of rice *NOE1*, a gene whose knockout enhances NO production, revealed that this is indeed the rice CAT OsCATC (Lin et al., 2011). Increase in leaf H_2O_2 content leads to a characterization of mutant *NOE1* which in turn leads to nitrate reductase (NR) dependent NO production. Increased H_2O_2 concentrations provoked by ABA may in turn trigger NO generation by NR and nitrogen oxide synthase (NOS)-like enzymes (Neill et al., 2008). NO accumulation under abiotic stress is similar to the events seen in H_2O_2 production (Wang et al., 2014). In *Arabidopsis*, both H_2O_2 and NO showed similar function which influences the induction and reduction in root growth stimulated by various concentrations of nucleotides (Clark et al., 2010). He et al. (2013) reported that NO and H_2O_2 are also involved in the stimulation of stomatal closure in *Arabidopsis* in response to ultraviolet-B exposure. Exclusion of H_2O_2 with antioxidants or inhibition of its synthesis by inhibiting NADPH oxidase activity prevents NO generation and stomatal closure. Wang et al. indicated the idea that H_2O_2-induced synthesis of NO might be mediated by MPK6 in *Arabidopsis* (Wang et al., 2010).

SALICYLIC ACID AND INTERACTION WITH H_2O_2

Salicylic acid is one of the key phytohormones involved in both abiotic (Kunihiro et al., 2011; Drzewiecka et al., 2012; Liu et al., 2012) and biotic (Vlot et al., 2008; Dempsey et al., 2011) stress adaptation. The discovery of the salicylate role in thermotolerance during potato tissue culture research was mere coincidence. Inclusion of the artificial SA analog acetyl salicylic acid (ASA) in the culture medium of microplants of potato (*Solanum tuberosum* L.) causes potentially useful effects such as tuberization. It has been shown to play a central role as a signaling molecule involved in both local defense reactions and in the induction of systemic resistance (Durner

and Klessig, 1999; Herrera-Vásquez et al., 2015). Another important aspect is gene regulation of antifungal hydrolases by SA, such as pathogenesis-related (PR) encoding PR1 and PR proteins which target to the plant cell wall (Durner and Klessig, 1999). Reduced synthesis of SA in transgenic plants due to disruption of SA pathways results in vulnerability toward fungal (*Phytophtora parasitica, Cercospora nicotianae*), bacterial (*Pseudomonas syringae*), and viral (*tobacco mosaic virus*) pathogens (Delaney et al., 1994).

There are various reports suggesting involvement of SA in various biotic and abiotic stresses (Wan et al., 2012; Miura and Tada, 2014; Herrera-Vásquez et al., 2015). Various environmental factors such as temperature, salinity, drought and high light exposure are responsible for ROS generation in cell organelles (peroxisomes, chloroplast) (Apel and Hirt, 2004; Holuigue et al., 2007). SA can be directly or indirectly involved in signaling pathways and interplays with ROS and GSH in stressed plants (Mateo et al., 2006; Lee and Park, 2010; Herrera-Vásquez et al., 2015). Under drought stress, increased level of SA has been observed in oat plants (Sánchez-Martín et al., 2015), whereas another study also stated the same condition in peroxisome and chloroplast for the gene *cat2* knockout (Chaouch et al., 2010) and thylakoidal ascorbate PRX gene silencing (Maruta et al., 2012; Noshi et al., 2012), respectively. H_2O_2 stimulated PCD, SA accumulation and sesquiterpene production in cultured cell suspensions of *Aquilaria sinensis* (Liu et al., 2014). H_2O_2 production induced SA up-regulated the mRNA transcription of heat shock protein (Hsp) genes through *AtHsfA2*, a key component of acquiring thermotolerance in *Arabidopsis* (Nie et al., 2015).

Salicylic acid and H_2O_2 interrelation is suggested by Leon, since the pathway involved benzoic acid (the immediate precursor of SA) leads to activation of benzoic acid 2-hydroxylase which is H_2O_2 dependent (León et al., 1995). Relative pathway of this case suggest that H_2O_2 production in cell organelles (peroxisome and chloroplast) induces SA synthesis, and leads to protective mechanism such as stomatal closure and cell death. Salicylate can increase H_2O_2 levels in plant tissues (Rao et al., 1997; Dat et al., 1998), on the contrary SA accumulation can be induced by elevated H_2O_2 levels (Chamnongpol et al., 1998). The germination of *sid2* seeds under high salinity is hypersensitive to H_2O_2, but the physiological concentrations of SA modulate antioxidant activity to prevent oxidative damage (Lee et al., 2010). There is another study suggesting exogenous application of SA relieves Cd toxicity by reducing the H_2O_2 accumulation in root apoplasts of the legumes *Phaseolus aureus* and *Vicia sativa* (Zhang et al., 2011).

The HR to pathogens exhibits an early 'oxidative burst' of superoxide which rapidly dismutates to H_2O_2. This mechanism involves key interactive roles for SA and H_2O_2, as the HR was impaired in tobacco plants with an H_2O_2-inducible SA-hydroxylase transgene (Mur et al., 1997). It is considered that mammalian plasma membrane NADPH oxidase is a homolog of oxidative stress enzyme (Keller et al., 1998) and it may be that this enzyme is potentiated by SA (Kauss and Jeblick, 1996). Increased accumulation of SA and enhancement in H_2O_2 concentration in simultaneous pathogenesis gene induction were observed in *GR1* dependent glutathione (Mhamdi et al., 2010).

ASCORBIC ACID AND INTERACTION WITH H_2O_2

Ascorbic acid (AsA) is a critical water soluble phytohormone found in plant and animals (Levine, 1986; Sies and Stahl, 1995). It acts as a signal for plant growth and development, and regulates cell division, growth and signal transduction (Kerk and Feldman, 1995; Smirnoff and Wheeler, 2000). In the mitochondria plants synthesize AsA which is then transported to other parts of the plants (Shao et al., 2008). There can be a direct or indirect reaction of H_2O_2 with AsA, which is catalyzed by APX. APX is responsible for scavenging H_2O_2 hyperaccumulation found in higher plants (cytosol, chloroplast and mitochondria) (Mittler and Zilinskas, 1991).

H_2O_2 detoxification can be done by various antioxidants in peroxisomes such as CAT in the matrix, APX and monodehydroascorbate reductase (MDAR) in association with AsA, resulting in a decrease in the accumulation of H_2O_2 (Yamaguchi et al., 1995; Karyotou and Donaldson, 2005). In the chloroplast stroma, where the pH is higher during the day time, there is a consequence of AsA consumption during H_2O_2 reduction. A rate limiting amount of dehydroascorbate reductase (DHAR) efficiently catalyzes the recycling of AsA. The signaling function of H_2O_2 in guard cells is controlled by the rate of its production and the rate of its removal, in which AsA and DHAR play a critical role. The slower responsiveness of guard cells of DHAR over expressing tobacco allows more ozone to diffuse into the leaf interior (Chen and Gallie, 2004). However, the increase of AsA content in all cells and consequent increase in their ability to detoxify entered ozone, reduce the oxidative load of the leaf (i.e., lower levels of foliar and apoplectic H_2O_2). From past reports, it is clear that the oxidative stress induced ROS level increases monodehydroascorbate (MDA) accumulation which is being converted into L-ascorbate (AsA) and dehydroascorbate (DHA) (**Figure 3A**). The accumulated H_2O_2 is reduced to H_2O by oxidation of AsA to MDA radical, which is catalyzed by APX. The MDHA is subsequently reduced back to AsA by either ferredoxin reduction or NAD(P)H catalyzed monodehydroascorbate reductase (MDHAR) (Sano et al., 2005). In GPX cycle, similar to APX, GPX uses GSH as a reducing agent to detoxify H_2O_2 to H_2O. In addition to GPX, the organellar redox state is regulated by different enzymatic antioxidants like GR, MDHAR in addition to GPX. Disproportionation in L-ascorbate and MDHA is maintained by GSH (Venkatesh and Park, 2014) (**Figure 3A**).

JASMONATE AND INTERACTION WITH H_2O_2

Phytohormones act as a major factor responsible for plant growth and development. Oxylipin is considered to be one of the most important signaling molecules, i.e., plant hormone JA. Due to

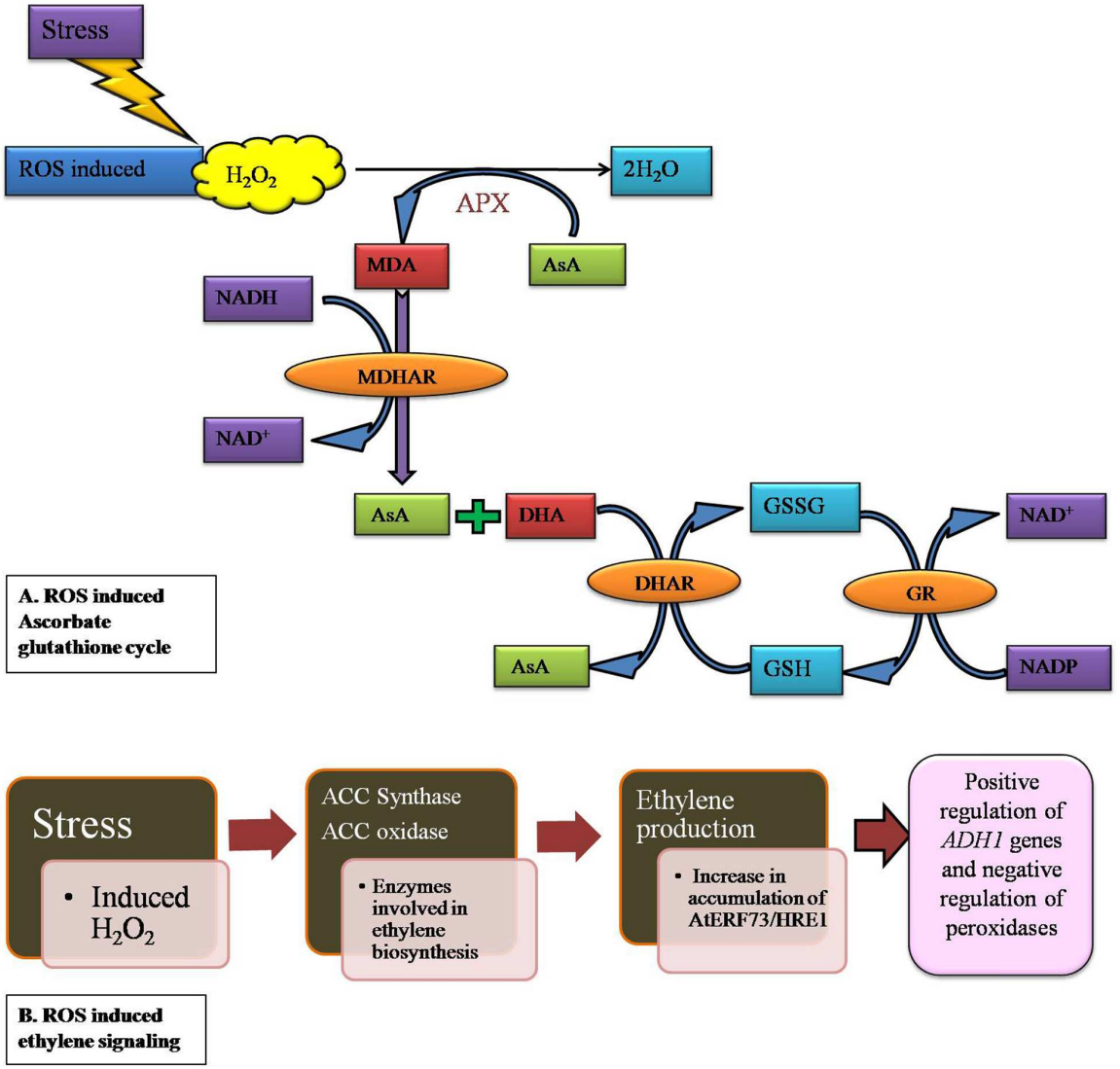

FIGURE 3 | H_2O_2 induced antioxidant mechanism in plants which involved in detoxification process. (A) Plants synthesize ascorbic acid in the mitochondria and transported to other parts of the plants. Ascorbate peroxidase (APX) uses AsA as a substrate to reduce H_2O_2 to H_2O in the ascorbate-glutathione cycle and generate mono dehydroascorbate (MHA), which further dissociate to AsA and dehydroascorbate (DHA). **(B)** Ethylene responsiveness in gene induction upon H_2O_2 accumulation in plant cells. Enzymes which involved in ethylene production activate an ethylene responsive group (AtERF73/HRE1). These genes are involved in negative regulation of peroxidases which in turn reduces H_2O_2 accumulation.

the unique physiological properties and abundance, jasmonate and its derivate (methyl jasmonate) came into the limelight as bioactive in nature. Chloroplast membrane is considered to be the initial site for JA synthesis where membrane phospholipids acts as a source for alfa-linolenic acid (C18:3) and hexadecatrienoic acid (C16:3) production (Ishiguro et al., 2001). Major pathway for JA synthesis in plants is supposed to be an octadecanoid pathway with the involvement of alfa-linolenic acid as a substrate (Mueller et al., 1993).

The defensive mechanism of JA has been observed in tomato against tobacco hornworm larvae (Howe et al., 1996) whereas in *Arabidopsis* against the fly *Bradysia* (McConn et al., 1997). There are studies confirming the role of JA as protective agent against pathogen (*Pythium mastophorum)* attack in *Arabidopsis* (Vijayan et al., 1998). Hu et al. (2003), came across with the result that both H_2O_2 and JA are primary signaling molecules during the cellular response involved in saponin biosynthesis mediated by oligogalacturonic acid (OGA) which also leads to the H_2O_2 mediated upregulation of JA. JA derivate (methyl jasmonate) has also been studied for its involvement in the induction of H_2O_2 accumulation in parsley suspension-cultured cells (Kauss et al., 1994), whereas another study suggested its role in inducing defensive genes of tomato (Orozco-Cardenas et al., 2001).

Jasmonic acid induces glutathione, an important antioxidant for redox balance. Increased expression of nuclear factor erythroid 2-related factor 2 (*NrF2*) has also been observed,

which reduces the ROS level induced by H_2O_2 (Taki-Nakano et al., 2014). In association with this study, increased expression of glutamyl cysteine ligase with an increase in *NrF2* helps in regulating enzymes reducing oxidative stress (Bea et al., 2003).

ETHYLENE AND INTERACTION WITH H_2O_2

Ethylene has long been regarded as a stress hormone (Morgan and Drew, 1997). It is not only involved in plant growth and development, but also involved in plant responses to biotic stress, such as pathogen attack; and abiotic stress, such as wounding, ozone, and salinity (Abeles et al., 1992; Wang et al., 2009). Ethylene regulates many different processes in plants and has shown response in defense mechanism as well (Ecker, 1995). In order to evaluate the defensive role of ethylene against various environmental conditions signal transduction pathways for ethylene has been studied with mutants.

The roles of ethylene have been established in damage control caused by virulent bacteria or fungal pathogens when it is being inoculated (Bent et al., 1992; Lund et al., 1998) but its importance against avirulent bacteria infected plants has yet to be proven (Bent et al., 1992). The most important signaling molecules in the ethylene pathway are *ETR1* and *EIN2* (Buer et al., 2006). Change in gene expression of the ethylene receptor (*ETR1*) results in reduced ethylene response (O'Malley et al., 2005).

Environmental stress affects many signaling pathways in plants which also includes an alternative pathway (AP). Despite slight evidence about H_2O_2 and ethylene playing roles in inducing AP, there is no clear picture of how these signaling molecules are inducing the AP under various environmental conditions. Results of Wang et al. (2010) showed the possibility of involvement of H_2O_2 and ethylene mediated induction of AP under salt stress as it shows activity in wild-type callus whereas no activity was observed with *ETR1-3* callus. In recent years, an increasing number of positive results on ethylene toward mutants in *Arabidopsis* have confirmed its role in signaling pathways (Guo and Ecker, 2004). In another study H_2O_2 accumulation in simultaneous production of ethylene has been observed in tobacco plant stressed with ozone (Schraudner et al., 1998). In plants, oxygen-deficient conditions shifts energy metabolism from aerobic to anaerobic, which in turn adversely affects nutrient and water uptake. Eventually, hypoxiasignalling triggers the production of both hydrogen peroxide (H_2O_2) and ethylene. H_2O_2 and ethylene interplay has an effect on *AtERF73/HRE1* and *ADH1* expression during the early stages of hypoxia signaling in *Arabidopsis*. Hypoxia signaling induces the ethylene biosynthesis enzymes such as *ACC synthase* (*ACS*) and *ACC oxidase* (*ACO*) (Peng et al., 2005) (**Figure 3B**). *Arabidopsis AtERF73/HRE1* is very similar to the rice *Sub1A* and *SNORKEL* genes, which belongs to the group VII ERF (ethylene responsive factor) subfamily. They play major roles in the submergence tolerance of lowland and deepwater rice (Hattori et al., 2009). According to Yang (2014), *AtERF73/HRE1* positively regulates *ADH1* genes as well as negatively regulates *peroxidase* and *cytochrome* P450 genes in hypoxia signaling (**Figure 3B**).

CONCLUSION

Increasing urge to identify the role of hydrogen peroxide as a signaling molecule has gathered the interest of researchers to focus their work on the mechanisms regulating the generation of hydrogen peroxide, and this is certainly an important growing area of research. Significant scientific effort in the last 10 years has determined the position of H_2O_2 in signal transduction networks in plants, demonstrating that it is essential for both the communication between external biotic and abiotic stimuli, and the control of developmentally regulated processes. There are many signaling pathways for H_2O_2 mediated stress and defense responses that have been studied, but it remains a large scope of additional research unexplored, which can further clarify the mechanism involved in these pathways. The focus should be imposed on a clear description of roles of endogenous compounds which modify the plant responses. It has been reported that the phytohormones like ABA, SA, JA, GA, and ethylene regulates the protective responses in plants under abiotic stress by involving in different H_2O_2 induced signaling. Despite of its regular activities in plant growth and development, ABA plays crucial role in H_2O_2 mediated stress cues. Zhang et al. (2007) indicated that ABA-induced H_2O_2 production mediates NO generation, which in turn, activates MAPK cascade and results in the over expression and up regulation in of antioxidant enzyme activities in ABA signaling. However, there are some contradictory roles of NO. According to Orozco-Cárdenas and Ryan (2002), NO has been shown to negatively modulate wound signaling in tomato plant blocking H_2O_2 production and proteinase inhibitor synthesis by JA, contradicting with previous study in which NO has been considered to show positive response in abiotic stress. Nevertheless, there are many studies suggesting H_2O_2 response in association with NO generation under biotic/abiotic stress (Delledonne et al., 2001; Romero-puertas and Sandalio, 2016).

Due to different results suggesting various roles of H_2O_2, it is important to focus future studies in getting a clear picture of signaling pathways during stress response in various conditions. Interactions between different signaling molecules and their biological functioning with the involvement in various pathways still needs to be cataloged. Another important aspect that should be focused on is the role and localization of enzymes, which are involved in signaling pathways. Some important factors for future research should be the identification of the site for H_2O_2 production in the cell and the major factors influencing its interaction with other signaling molecules.

AUTHOR CONTRIBUTIONS

ZC initiated the project. SS produced the figures. IS, SS, and ZC wrote the manuscript. SS and ZC revised the manuscript.

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Brassinosteroid Ameliorates Zinc Oxide Nanoparticles-Induced Oxidative Stress by Improving Antioxidant Potential and Redox Homeostasis in Tomato Seedling

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In the last few decades use of metal-based nanoparticles (MNPs) has been increased significantly that eventually contaminating agricultural land and limiting crop production worldwide. Moreover, contamination of food chain with MNPs has appeared as a matter of public concern due to risk of potential health hazard. Brassinosteroid has been shown to play a critical role in alleviating heavy metal stress; however, its function in relieving zinc oxide nanoparticles (ZnO NPs)-induced phytotoxicity remains unknown. In this study, we investigated the potential role of 24-epibrassinolide (BR) in mitigating ZnO NPs-induced toxicity in tomato seedlings. Seedling growth, biomass production, and root activity gradually decreased, but Zn accumulation increased with increasing ZnO NPs concentration (10–100 mg/L) in growth media (1/2 MS). The augmentation of BR (5 nM) in media significantly ameliorated 50 mg/L ZnO NPs-induced growth inhibition. Visualization of hydrogen peroxide (H_2O_2), and quantification of H_2O_2 and malondialdehyde (MDA) in tomato roots confirmed that ZnO NPs induced an oxidative stress. However, combined treatment with BR and ZnO NPs remarkably reduced concentration of H_2O_2 and MDA as compared with ZnO NPs only treatment, indicating that BR supplementation substantially reduced oxidative stress. Furthermore, the activities of key antioxidant enzymes such as superoxide dismutase (SOD), catalase, ascorbate peroxidase and glutathione reductase were increased by combined treatment of BR and ZnO NPs compared with ZnO NPs only treatment. BR also increased reduced glutathione (GSH), but decreased oxidized glutathione (GSSG)] and thus improved cellular redox homeostasis by increasing GSH:GSSG ratio. The changes in relative transcript abundance of corresponding antioxidant genes such as *Cu/Zn SOD, CAT1, GSH1,* and *GR1* were in accordance with the changes in those antioxidants under different treatments. More importantly, combined application of BR and ZnO NPs significantly decreased Zn content in both shoot and root of tomato seedlings as compared with

ZnO NPs alone. Taken together, this study, for the first time, showed that BR could not only improve plant tolerance to ZnO NPs but also reduce the excess zinc content in tomato seedlings. Such a finding may have potential implication in safe vegetable production in the MNPs-polluted areas.

Keywords: brassinosteroids, food safety, hydrogen peroxide, nanotoxicity, oxidative stress, tomato, zinc oxide nanoparticles

INTRODUCTION

Nanoparticles (NPs) are particles that have at least one dimension less than 100 nm; but have a greater surface area compared to bulk products. In the recent years, engineered NPs are extensively being used for manufacturing variety of industrial, commercial, and medical products that are eventually released to the environment. Thus extensive use of NPs has become a matter of public concern due to potential contamination of food chain by metal-based NPs (MNPs). Zinc oxide NPs (ZnO NPs) are one of the MNPs that are commonly investigated with regards to human and ecosystem health as well as nanotoxicological effect on plants (Chen et al., 2015; Van Aken, 2015; Zhang et al., 2015). Given that ZnO NPs are wide band-gap semiconductors that exhibit near UV emission and transparent conductivity, their application as electronic sensors and solar voltaic has become common (Lin and Xing, 2008; Ma et al., 2013). Moreover, ZnO NPs are widely exploited for their photolytic properties and are also extensively used in personal care products for their ultraviolet-blocking ability (Hoffmann et al., 1995; Ali et al., 2008). Earlier studies have already reported the presence of ZnO NPs in sewage treatment plant effluents and in sludge-treated soils used for agriculture (Ma et al., 2013). Another potential formulation of ZnO NPs for agricultural applications could be as a pesticide because of their antimicrobial properties (He et al., 2011; Dimkpa et al., 2013).

Earlier studies on nanotoxicity in plants showed differential effects of MNPs that include positive, negative or no effects on plants (Dietz and Herth, 2011). Nonetheless, most of those studies addressed easily observable parameters, such as germination rate and growth-related features. Plant responses to MNPs not only depend on dose, but also on the species of plants (Chichiriccò and Poma, 2015). For instance, ZnO NPs caused a dose-dependent inhibition in seed germination of cabbage (*Brassica oleracea* var. *capitata* L.), while it showed no negative effects on germination of maize (*Zea mays* L.) seeds (Pokhrel and Dubey, 2013). Moreover, ZnO NPs (1,000 mg/L) reduced root length of corn and cucumber (*Cucumis sativus* L.), but exhibited no effects on their seed germination (Zhang et al., 2015). In comparison with Zn^{2+}, toxicity of ZnO NPs on the root elongation of corn could be attributed to the nanoparticulate ZnO, while released Zn ion from ZnO could solely contribute to the inhibition of root elongation in cucumber (Zhang et al., 2015).

On the basis of NP size, shapes, compositions and atomic arrangement, interaction of NPs with cellular structure varies a lot and yet very complex and poorly understood (Van Aken, 2015). Moreover, studies on the mechanism of interaction between NPs such as ZnO NPs and plant cell biomolecules are scanty. A few studies on NPs ecotoxicity suggested several potential mechanisms through which ZnO NPs cause damage to plants (Chen et al., 2015; Zhang et al., 2015). MNPs enter plant tissues, predominantly through pores in the cell wall and endocytosis pathway, and then are translocated through vascular system (Chichiriccò and Poma, 2015). First, the release of Zn^{2+} from ZnO NPs possibly causes phytotoxicity (Lin and Xing, 2008). Second, ZnO NPs due to their small sizes and large surface area may directly interact with biomolecules and disrupt membranes or DNA (Ma et al., 2013). Most importantly, ZnO NPs promote the generation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) in the absence of photochemical energy (Hernandez-Viezcas et al., 2011). It is suggested that excessive generation of ROS can induce membrane lipid peroxidation and cellular damage, which is considered as one of the primary reasons contributing to nanotoxicity in plants (Chichiriccò and Poma, 2015). Plants possess a well equipped antioxidant systems composed of both enzymatic and non-enzymatic antioxidants to scavenge excessive level of ROS that are injurious to cells (Baxter et al., 2014). Exposure of plants to NPs also stimulates antioxidant system in plants, perhaps as an adaptive response to alleviate oxidative stress.

In general, Zn is required for various metabolic processes in human. However, daily intake that exceeds recommended dietary allowance may cause Zn toxicity (Fosmire, 1990). Ingestion of excess Zn causes symptoms like nausea, vomiting, epigastric pain, abdominal cramps, and diarrhea in human. Furthermore, the use of Zn supplements can interfere with the utilization of copper, impair immune function and severely affect lipoprotein profiles (Fosmire, 1990). Vegetables are an integral part of human diet that serves as a major route for intake of essential and/or non-essential metals. An earlier study conducted in a metal-polluted area (Zinc Plant in Huludao City, China) showed high transfer factor values of Cd, Zn, and Cu from soil to vegetable (Zheng et al., 2007). Authors of that study calculated total metal target hazard quotients due to consumption of vegetables, which indicated highest health risks to inhabitants close to Zinc plant. Thus, extensive utilization of ZnO NP and deliberate release to environment may increase possible health risks to population of metal-polluted areas through the food chain transfer. Therefore, elucidation of strategies that improve plant tolerance to MNP as well as reduce metal uptake are urgent to ensure food security and food safety. Till date, studies relating to alleviation of ZnO NPs-induced phytotoxicity are scanty. Recently, Chen et al. (2015) reported that nitric oxide (NO), a ubiquitous signal molecule in plants could ameliorate ZnO NPs-induced phytotoxicity in rice seedlings. A number of earlier studies

relating to enhancement strategies of plant tolerance against heavy metal stress (not MNPs) revealed that signal molecule including phytohormones could alleviate heavy metal-induced phytotoxicity in a range of plant species (Ahammed et al., 2014). For instance, (Ramakrishna and Rao, 2012, 2015) reported that 24-epibrassinolide (a steroidal phytohormone) could alleviate Zn^{2+} stress by improving antioxidant potential and redox state in radish (*Raphanus sativus* L.) seedlings. ZnO NPs can also induce Zn^{2+} stress by releasing Zn ions. Thus it is quite likely to anticipate a protective role of brassinosteroids (BRs) against MNPs too.

Brassinosteroids are a class of plant-specific essential steroidal hormones that regulate broad aspects of plant growth, development and responses to various biotic and abiotic stresses (Cui et al., 2011; Ahammed et al., 2014; Zhou et al., 2014; Divi et al., 2016). However, the mechanisms that control BRs-induced stress tolerances are largely unknown (Divi et al., 2016). We previously showed that BRs-induced oxidative stress tolerance involves transient accumulation of hydrogen peroxide (H_2O_2) that activates antioxidant system in cucumber and tomato plants (Cui et al., 2011; Nie et al., 2013; Zhou et al., 2014). Although BRs initially induce NADPH-based H_2O_2 production, it eventually enhances ROS scavenging by stimulating antioxidative machinery, indicating a dual role of H_2O_2 in mediating BR-induced stress tolerance (Ahammed et al., 2014). We also showed that BRs could efficiently ameliorate cadmium (a major toxic heavy metal)-induced oxidative stress and photosynthetic inhibition in tomato plants (Ahammed et al., 2013). However, the effect of BRs on MNPs-induced phytotoxicity still remains elusive. In the current study, we investigated the potential role of 24-epibrassinolide (BR, a bioactive BRs) in mitigating ZnO NPs-induced toxicity in tomato seedlings. This study is expected to provide a better insight into the role of BRs in MNPs-induced oxidative stress that may be useful to ensure food safety in MNPs-polluted areas.

MATERIALS AND METHODS

MNPs Preparation

The ZnO NPs (diameters varying between 20 and 30 nm, a purity > 99%) were purchased from the Aladdin Corporation (Shanghai, China). Culture dispersion of NPs was achieved by adding Phytagel (Sigma–Aldrich, St. Louis, MO, USA) powder and a suitable amount of NPs to ultrapure water, and the dispersions were sufficiently shaken after sonication to break up agglomerates. Each concentration of NPs treatment was prepared separately, without dilution, by weighing particles and dispersing them in solid half strength Murashige and Skoog (½ MS) medium. The addition of NPs ranged from 10 to 100 mg/L. It is noteworthy to mention that the agar culture medium has the advantage of easy dispersion of NPs without precipitation.

Plant Materials and Treatments

Authentic tomato seeds (*Solanum lycopersicum* L. cv. Hezuo903)were sterilized in a 10% sodium hypochlorite solution for 15 min, rinsed thoroughly with deionized water several times, and subsequently placed in sterilized solid ½ MS medium at a controlled temperature of 28°C in the dark. After 48 h, the seeds were checked for the germination, and seeds that had sprouted were used in the test. The toxicity tests were conducted in a tissue culture bottle (240 mL). Each test unit contained 40 mL of ½ MS culture media with a specific concentration of ZnO NPs. Ten tomato seedlings were placed just above the surface of the medium of the test units. The test units were placed in a sterile room. After an incubation period of 15 days, the plants were separated from the agar media, and seedling growth was measured. The NP concentrations of 0, 10, 20, 50, and 100 mg/L were prepared in four replicate test units per treatment.

In the BR treatment, the 5 nM 24-epibrassinolide was added in the ½ MS medium in the process of the solidification. The concentration of BR was chosen based on preliminary dose trial (data not shown).

Measurement of Morphological Parameters and Root Activity

Morphological parameters such as fresh weight and length of shoot and root, and root activity were determined following exposure of tomato seedlings to different levels of ZnO NPs for 15 days. In each replicate, 10 plants were selected randomly, and length and fresh weight of each plant were determined. Average values of these 10 plants were considered as one replicate. For determination of root activity, three replicates for each ten treatments such as CK, 10, 20, 50, and 100 mg/L ZnO NPs were selected. Roots were washed thoroughly with distilled water and finally with deionized water and cut into small pieces of 3–4 mm. A 0.5 g portion of these roots sample was placed into tube; 5 mL 0.4% TTC (triphenyl tetrazolium chloride) and 5 mL 0.1 mM phosphatic buffer solution (pH 7.0) were added to the tube and allowed to react for 2 h at 37°C. Afterward, 2 mL of 1 M H_2SO_4 was added to the tube to stop the reaction. The root activity was expressed by the amount of TPF (triphenyl formazan) deoxidized by TTC (Islam et al., 2007).

Determination of H_2O_2 Contents and Histochemical Detection of H_2O_2

To determine the H_2O_2 concentration, 0.3 g of fresh root tissues was homogenized in 3 mL of precooled $HClO_4$ (1.0 M) using a pre-chilled mortar and pestle, according to the method of Willekens et al. (1997).

H_2O_2 production in root tissues was monitored using 2,7-dichlorofluorescein diacetate (H_2DCF-DA) as described by Yi et al. (2015). Detached roots were washed with deionized water and incubated 15 min with 25 μM H_2DCF-DA in 200 mM phosphate buffer (pH 7.4) and then washed five times with the same buffer without the dye. To scavenge H_2O_2, the root segments were incubated with 1 mM ascorbate or 100 U/mL catalase for 30 min that served as negative controls. Fluorescence was observed using a Leica DM4000B microscope and images were captured using a Leica DFC425C camera and the Leica

application suite V3.8 software (Leica Microsystems, Wetzlar, Hessen, Germany).

Determination of Lipid Peroxidation

To determine level of lipid peroxidation in roots, concentration of malondialdehyde (MDA) was measured by the 2-thiobarbituricacid (TBA) test. Root samples (0.5 g) were homogenized in 5 mL of 10% (w/v) trichloroaceticacid (TCA). The homogenates were centrifuged at 3,000 *g* for 10 min and 4 mL of 20% TCA containing 0.65% (w/v) TBA was added to 1 mL of the supernatant. The mixtures were heated in a hot water bath at 95°C for 25 min and immediately placed in an ice bath to stop the reaction. After centrifugation at 3,000 *g* for 10 min and the absorbance of the supernatants was recorded at 440, 532, and 600 nm. The MDA equivalents were calculated according to Hodges et al. (1999).

Determination of Antioxidant Enzyme Activity

Antioxidant enzymes were extracted by grinding the root tissue (0.3 g) with 3 mL ice-cold 50 mM phosphate buffer (pH7.8) containing 0.2 mM EDTA and 2% polyvinylpyrrolidone (w/v). The homogenates were centrifuged at 4°C for 20 min at 12,000 *g*, and the resulting supernatants were used for the determination of enzymatic activity.

Superoxide dismutase (SOD) activity was assayed by inhibiting the photochemical reduction of nitro blue tetrazolium (NBT) according to Stewart and Bewley (1980). The absorbance was monitored in 560 nm. One unit of SOD is the amount of extract that gives 50% inhibition of the reduction rate of NBT. Catalase (CAT) activities were determined following the methods of Cakmak and Marschner (1992). The reaction mixture for CAT consisted of 25 mM phosphate buffer (PH 7.0), 10 mM H_2O_2 and enzyme extract. The decomposition of H_2O_2 determined at 240 nm for 20 s (E_{240} = 39.4 mM^{-1} cm^{-1}). Ascorbate peroxidase (APX) was measured in a reaction mixture containing 25 mM phosphate buffer (PH 7.0), 5 mM ascorbic acid (ASA), 20 mM H_2O_2 and enzyme extract with the principle of monitoring the rate of ascorbate oxidation at 290 nm for 20 s (E_{290} = 2.8 mM^{-1} cm^{-1}), according to Nakano and Asada (1981). Glutathione reductase (GR) activity was determined according to Foyer and Halliwell (1976). The reaction mixture for GR contained 25 mM phosphate buffer (PH 7.0), 10 mM glutathione and oxidized glutathione (GSSG), 2.4 mM NADPH and enzyme extract. The detection was based on the rate of decrease in the absorbance of NADPH at 340 nm for 20 s (E_{340} = 6.2 mM^{-1} cm^{-1}). All of the spectrophotometric analyses were performed using a SHIMADZU UV-2410PC spectrophotometer (Japan).

Determination of Glutathione

Glutathione contents were determined according to Sgherri and Navari-Izzo (1995). Root tissues (0.3 g) were homogenized in 2 mL of 6% metaphosphoricacid containing 2 mM EDTA. The homogenates were then centrifuged at 4°C for 10 min at 14,000 *g*. The total GSSG contents were determined using the 5, 5′dithio-bis (2-nitrobenzoicacid)-GSSG reductase recycling method. The reaction mixture for total glutathione (GSH+GSSG) consisted of 100mM phosphate buffer (PH 7.5), 6 mM 5, 5′dithio-bis (2-nitrobenzoicacid; DTNB), 0.2 mM NADPH, three units of GR and extract with the principle of monitoring the decomposition of DTNB at 412 nm for 1 min. For detection of GSSG, GSH in the extract was blocked out by adding 2-ethenylpyridine, and then measured following the same procedure of total glutathione. Reduced glutathione (GSH) content was then calculated by deducting GSSG from total glutathione (GSH+GSSG).

RNA Isolation and RT-PCR

Plant root samples were ground in liquid nitrogen and the total RNA was isolated according to the manufacturer's protocol using the Trizol reagent (Invitrogen, California, CA, USA). The genomic DNA was removed using the RNeasy Mini Kit (Qiagen, Beijing, China). Total RNA (1 μg) was reverse-transcribed using the ReverTra Ace qPCR RT Kit (Toyobo, Japan) following the manufacturer's instructions. Gene-specific primers for the quantitative real time PCR (qRT-PCR) were designed based on the mRNA or EST sequences for the corresponding genes as follows: *Cu/Zn-SOD* (F: 5′-GGCCAATCTTTGACCCTTTA-3′, R: 5′-AGTCCAGGAGCA AGTCCAGT-3′), *cAPX* (F: 5′- TCTGAATTGGGATTTGCTGA-3′, R: 5′-CGTCTAACGTAGCTGCCAAA-3′), *GR1* (F: 5′-TTG GTGGAACGTGTGTTCTT-3′, R: 5′-TCTCATTCACTTCCC ATCCA-3′), *CAT1* (F: 5′-TGATCGCGAGAAGATACCTG-3′, R: 5′-CTTCCACGTTCATGGACAAC-3′), and *GSH1* (F: 5′-CTGC ATTCTGGGTGGGT-3′, R: 5′-CTCGGCTACTTCGTTCA-3′); *Actin* (F: 5′-TGGTCGGAATGGGACAGAAG-3′, R: 5′-CTCAGTCAG-GAGAACAGGGT-3′) was used as an internal control. For the qRT-PCR, the PCR products were amplified in triplicate using the SYBR Green PCR Master Mix (Takara, Tokyo, Japan) in 25 μL qRT-PCR reactions in an iCycler iQ^{TM} 96-well real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The PCR conditions consisted of denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. The software that was provided with the PCR system was used to calculate the threshold cycle values and quantify the mRNA levels according to Livak and Schmittgen (2001).

Determination of Zn Content

Dry samples (0.10 g) of shoot or root (homogenized and powdered) were digested with a mixture of $HClO_4$ and HNO_3 (v/v = 1/9) at 180°C. The digested colorless liquids were washed three times with distilled water. The liquid was collected and transferred to 50 mL volumetric flasks and diluted to a constant volume. Total Zn concentration was analyzed using an atomic absorption spectrophotometer (AA-6300; Shimadzu Co. Kyoto, Japan) as described by Wu et al. (2005).

Statistical Analysis

At least four independent replicates were used for each determination, and the mean values of all of the data are presented for each treatment. A statistical analysis of the

bioassays was performed with the SPSS 16.0 statistical software package, and a Tukey's test ($P < 0.05$) was performed to evaluate the treatment effects.

RESULTS

Dose Effect of ZnO NPs on Growth and Root Viability of Tomato Seedlings

To assess toxic effect of ZnO NPs on seedling growth, we first carried out a dose-trial of ZnO NPs by exposing tomato seedlings to different levels of ZnO NPs (10, 20, 50, and 100 mg/L) for 15 days. As shown in **Figures 1A,B**, both fresh biomass and length of shoot and root of tomato seedlings decreased significantly with increasing concentration of ZnO NPs in growth media. In addition, the root activity also reduced gradually with an increase in the concentration of ZnO NP (**Figure 1C**). To understand potential link between exposure concentration of ZnO NPs and subsequent growth inhibition, we quantified Zn content in tomato seedlings. Data showed that Zn content consistently increased with increasing concentration of ZnO NPs in media (**Figure 1D**), indicating that Zn has been absorbed by plant from media in a dose-dependent manner that caused substantial growth inhibition in tomato seedlings.

BR Improves Seedling Growth and Biomass Accumulation under ZnO NPs Stress

To investigate potential role of BR in tomato ZnO NPs tolerance, we selected two concentrations of ZnO NPs (10 and 50 mg/L) for further study, considering them as low and high dose of ZnO NPs based on our preliminary experiment. As shown in **Figure 2** both low and high concentration of ZnO NPs caused significant reduction in biomass accumulation accounting for 20.7/11.9 and 55.2/46.3% reduction in shoot/root, respectively (**Figure 2A**). Likewise, length of shoot and root decreased by 71.6 and 32.3%, respectively, following exposure of seedlings to 50 mg/L ZnO NPs (**Figure 2B**). In contrast, combined treatment of BR (5 nM) and

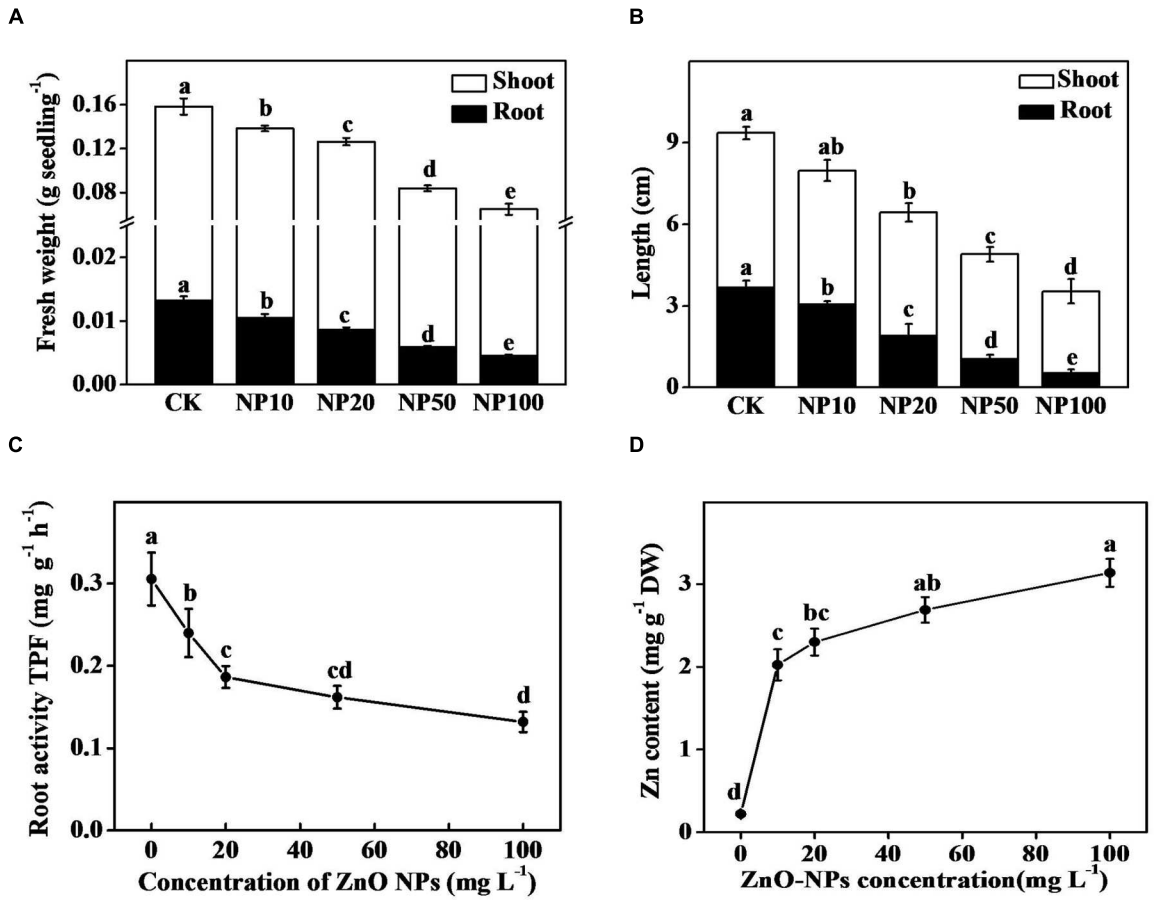

FIGURE 1 | Effect of different concentration of zinc oxide nanoparticles (ZnO NPs) on growth and biomass accumulation in tomato seedlings. **(A)** Fresh weight, **(B)** length of shoot and root, **(C)** root activity, and **(D)** Zn content in seedlings. Tomato seeds after sprouting were placed on ½ MS medium containing graded levels ZnO NPs and cultured for 15 days in tissue culture bottle. Data are the means of four replicates, with standard errors indicated by the vertical bars. Means denoted by the same letter did not significantly differ at a $P < 0.05$, according to Tukey's test. CK, control; DW, dry weight; NP10, 10 mg/L ZnO NPs; NP20, 20 mg/L ZnO NPs; NP50, 50 mg/L ZnO NPs; NP100, 100 mg/L ZnO NPs; TPF, triphenyl formazan.

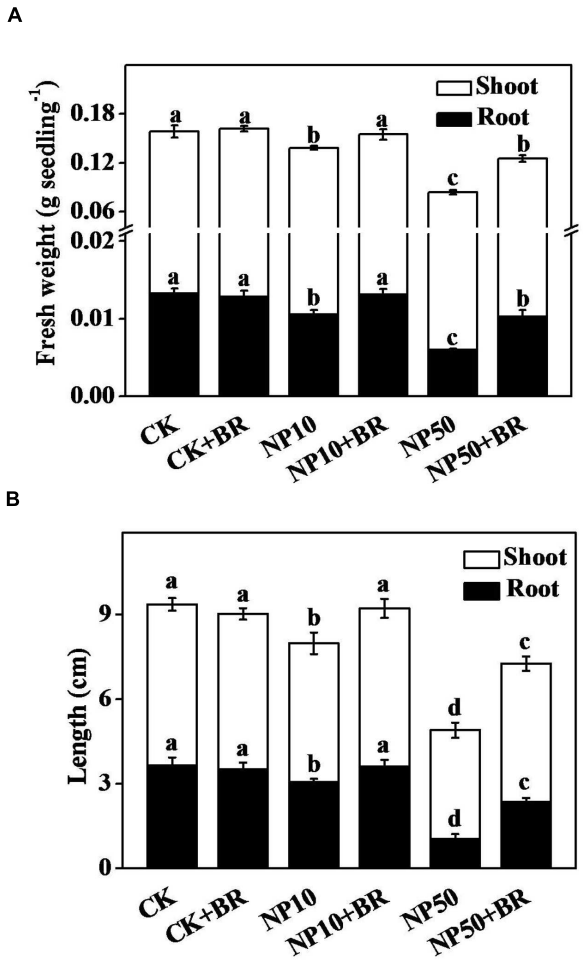

FIGURE 2 | Effect of brassinosteroid on growth and biomass accumulation under zinc oxide nanoparticles (ZnO NPs) stress. **(A)** Fresh weight, and **(B)** Length of shoot and root of tomato seedlings after 15 days culture on the ½ MS culture medium containing 5 nM 24-epibrassinolide (BR) and/or 10 and 50 mg/L ZnO NPs. The data are the means of four replicates, with standard errors indicated by the vertical bars. Means denoted by the same letter did not significantly differ at a $P < 0.05$, according to Tukey's test. NP10, 10 mg/L ZnO NPs; NP50, 50 mg/L ZnO NPs.

ZnO NPs remarkably increased fresh weight and length of shoot and root compared with those of ZnO NPs (both 10 and 50 mg/L) only treatments (**Figure 2**). For instance, BR treatment with 50 mg/L ZnO NPs increased fresh weight and length (shoot/root) by 72.4/47.7 and 126.9/26.9%, respectively, compared with those of 50 mg/L ZnO NPs alone. These observations clearly indicate that exogenous BR (5 nM) can alleviate both low and high concentration of ZnO NPs-induced inhibition of growth and biomass accumulation in tomato seedlings.

BR Minimizes ZnO NPs-Induced H_2O_2 Accumulation and Lipid Peroxidation

To understand potential mechanism of ZnO NPs-induced phytotoxicity, we examined H_2O_2 accumulation and lipid peroxidation that are often considered as reliable biomarkers of oxidative state. Hitochemical staining with H_2DCF-DA showed that exposure of seedlings to both concentration of ZnO NPs increased H_2O_2 accumulation in roots as evident by remarkably increased green fluorescence compared with that of control (**Figure 3A**). Biochemical analysis of H_2O_2 showed that H_2O_2 concentration increased by 27.8 and 66.0% in 10 and 50 mg/L ZnO NPs treatment, respectively, compared with that of control (**Figure 3B**). When BR was added with ZnO NPs in media, accumulation of H_2O_2 was reduced significantly compared with that of ZnO NPs only treatments (**Figure 2B**). Interestingly, BR application on non-stressed plants (CK) had no significant effect on H_2O_2 accumulation in roots of tomato seedlings (**Figure 2B**). We also quantified the MDA content to evaluate level of lipid peroxidation. In accordance with H_2O_2 accumulation, ZnO NPs (low and high) significantly increased MDA content in roots (**Figure 3C**). Compared with ZnO NPs only treatments, MDA content was remarkably decreased when BR was added with ZnO NPs in media, indicating that BR could efficiently minimize lipid peroxidation by lowering ROS accumulation in roots.

BR Improves Antioxidant Enzyme Activities and Redox Homeostasis under ZnO NPs Stress

Responses of antioxidant enzymes to individual and combined treatment of BR and ZnO NPs have been shown in **Figure 4**. Among four analyzed antioxidant enzymes such as SOD, CAT, APX, and GR, 10 mg/L ZnO NPs increased activities of CAT and GR only, while 50 mg/L ZnO NPs significantly induced activities of all four enzymes. For instance, activities of SOD, CAT, APX, and GR increased by 67.4, 85.8, 40.3, and 747.1% following exposure of seedling to 50 mg/L ZnO NPs for 15 days. Although BR treatment alone increased activity of GR only, combined treatment of BR and ZnO NPs remarkably increased activities of all four enzymes, compared with that of ZnO NPs (low and high) only treatments. These observations further confirm that alleviation of ZnO NPs-induced oxidative stress, is closely associated with enhancement of antioxidant enzyme activity by BR.

In addition, we assessed cellular redox state by analyzing GSH and GSSG contents, and comparing their ratio under different treatments in roots. Compared with the control, high concentration of ZnO NPs increased the contents of GSH, GSSG and GSH+GSSG, but decreased the GSH:GSSG ratio, whereas low concentration of ZnO NPs also increased contents of GSH and GSSG but had no significant effect on the GSH:GSSG ratio. Interestingly, combined treatment with BR and ZnO NPs significantly increased GSH, but decreased GSSG content, resulting in an increased GSH:GSSG ratio compared with that of ZnO NPs only treatments (both low and high). For example, compared with 50 mg/L ZnO NPs only treatment, combined treatment of BR and ZnO NPs increased GSH by 31.9%, but decreased GSSG by 48.5% which eventually increased GSH:GSSG ratio by 156.3%. BR only treatment also significantly increased GSH (32.0%) and decreased GSSG (15.0%), which ultimately increased the GSH:GSSG ratio (55.4%) compared with the

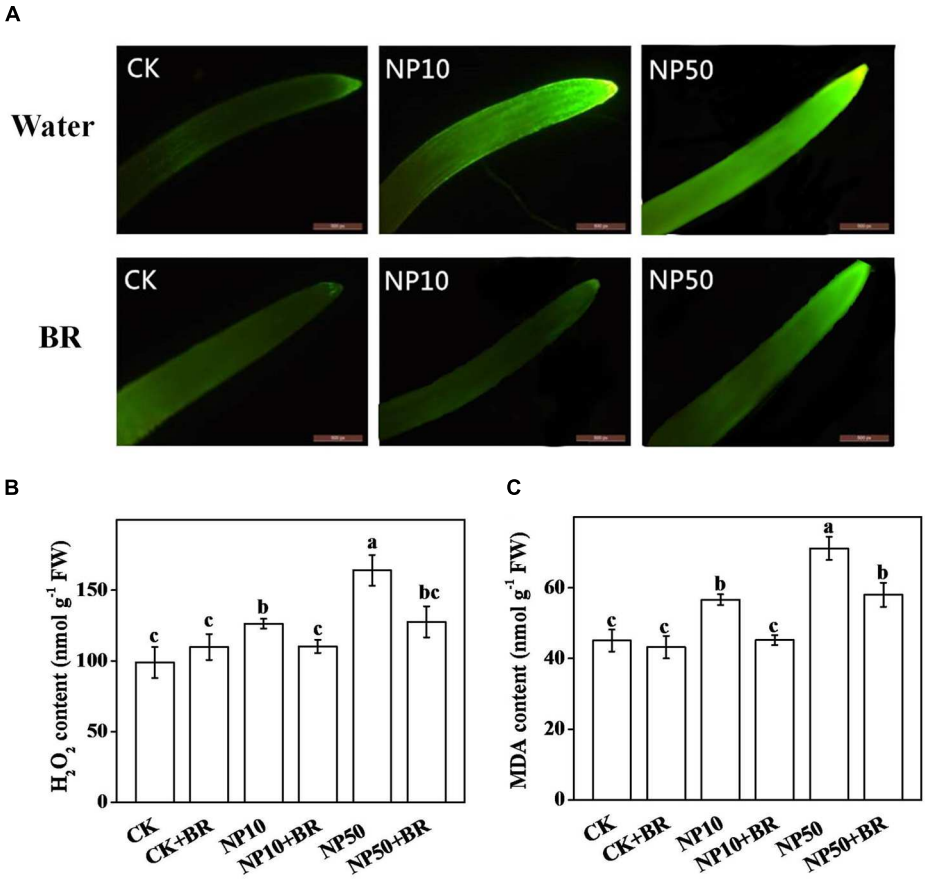

FIGURE 3 | H_2O_2 accumulation and lipid peroxidation in response to brassinosteroid and/or zinc oxide nanoparticles (ZnO NPs) treatments. (A) *In situ* detection of H_2O_2 using H2DCF-DA staining, Bar = 200 μm, **(B)** H_2O_2 contents, and **(C)** malondialdehyde (MDA) contents in seedling roots after 15 days culture in the ½ MS medium containing 10 and 50 mg/L ZnO NPs with or without 5 nM 24-epibrassinolide (BR). The data are the means of four replicates, with standard errors indicated by the vertical bars. Means denoted by the same letter did not significantly differ at a $P < 0.05$, according to Tukey's test. CK, control; FW, fresh weight; NP10, 10 mg/L ZnO NPs; NP50, 50 mg/L ZnO NPs.

control. These results clearly indicate that BR-induced alteration in glutathione helped young tomato seedlings to maintain redox homeostasis under ZnO NPs stress.

BR Upregulates Transcripts of Various Antioxidant-Related Genes under ZnO NPs Stress

To assess changes in transcripts of antioxidant genes under BR and/or ZnO NPs treatment, we assayed the expression of *Cu/ZnSOD*, *CAT1*, *GSH1,* and *GR1* genes in tomato roots. The transcript levels of *Cu/ZnSOD*, *CAT1*, *GSH1,* and *GR1* genes were all induced by both low and high ZnO NPs treatments (**Figure 6**). For example, 50 mg/L ZnO NPs treatment upregulated transcripts of *Cu/ZnSOD*, *CAT1*, *GSH1,* and *GR1* genes by approximately 1.5, 2.5, 1.8, and 1.9-fold, respectively. Importantly, addition of BR in media further increased the transcript levels of those genes. Thus, highest levels of transcript abundance were observed when seedlings were treated with BR and 50 mg/L ZnO NPs. BR-only treatment also upregulated transcripts of all four genes over control treatment, indicating a positive regulatory role of BR in transcription of those antioxidant-related genes.

BR Reduces Zn Accumulation under ZnO NPs Stress

To investigate whether BR-induced alleviation of ZnO NPs stress is also associated with changes in Zn accumulation, we quantified Zn content in shoot and root of tomato seedlings following exposure of seedlings to different treatments for 15 days. Zn accumulation in root was much higher than that in shoot under all treatments. In addition, Zn contents in shoot and root of BR only treated seedlings were not different from that of CK seedlings. However, exposure of seedlings to low and high ZnO NPs significantly increased Zn content both in shoot and root (**Figure 7**). The accumulation of Zn was very high in 50 mg/L ZnO NPs treatment, accounting for approximately 9.9 and 5.2-fold higher in shoot and root, respectively, compared with that of CK. Interestingly, Zn accumulation significantly reduced in roots and shoots when BR was applied with ZnO NPs. BR treatment decreased Zn content by 17.9 and 21.4% in shoots and roots,

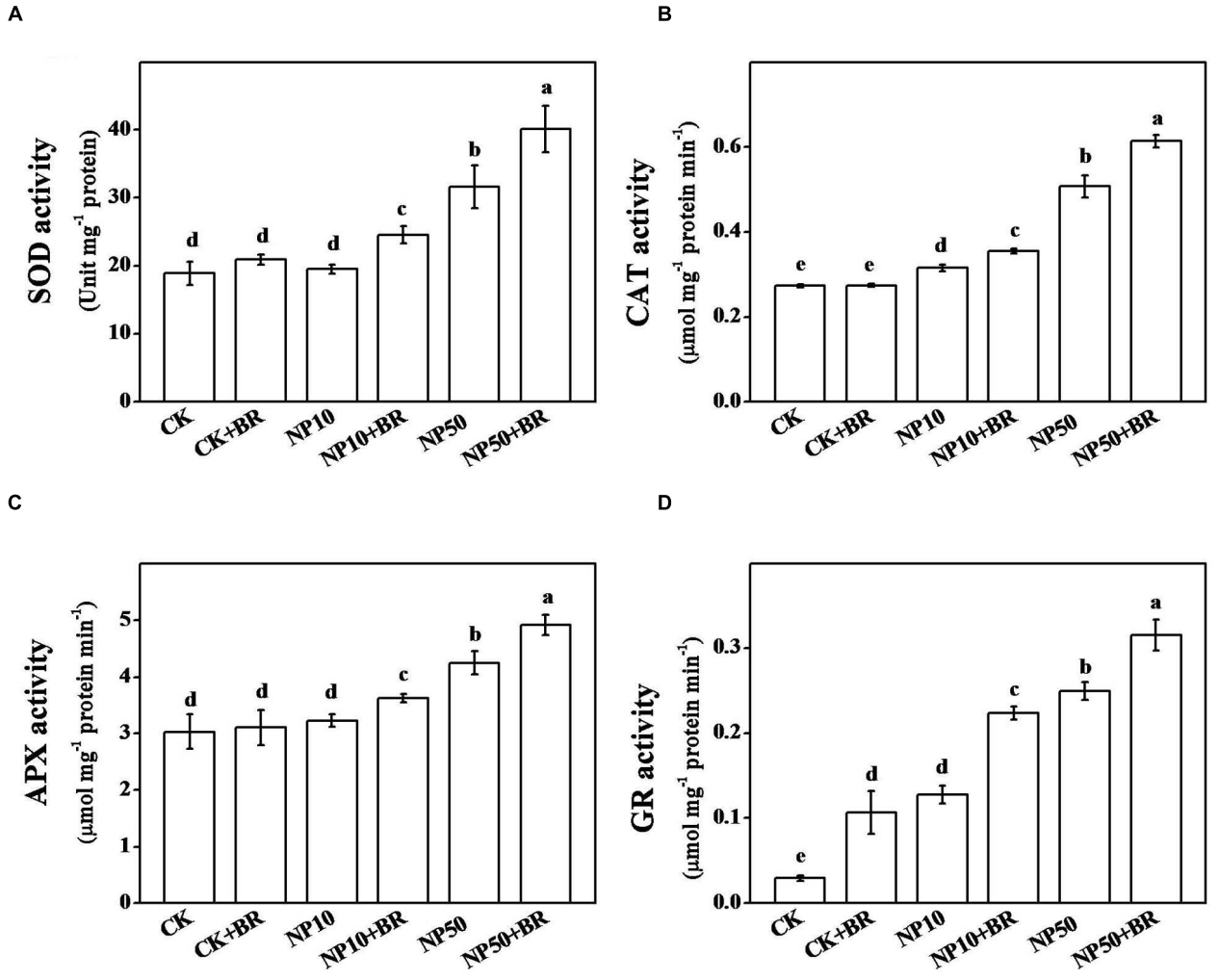

FIGURE 4 | Response of antioxidant enzymes to individual or combined treatment of brassinosteroid and zinc oxide nanoparticles (ZnO NPs). **(A)** Superoxide dismutase (SOD), **(B)** Catalase (CAT), **(C)** Ascorbate peroxidase (APX), and **(D)** Glutathione reductase (GR) activity in tomato roots after 15 days treatment. Tomato seeds after sprouting were placed on ½ MS medium containing 5 nM 24-epibrassinolide (BR) and/or 10 and 50 mg/L ZnO NPs (NP10 and NP50, respectively) and cultured for 15 days before the analysis of activities of antioxidant enzymes. The data shown here are the averages of four replicates, with the standard errors indicated by the vertical bars. The means denoted by the same letter did not significantly differ at a $P < 0.05$, according to Tukey's test.

respectively, after exposure to 10 mg/L ZnO NPs, while combined treatment of BR with 50 mg/L ZnO NPs reduced Zn content by 25.4 and 17.3% in shoots and roots, respectively, compared with that in ZnO NPs-only treatment (**Figure 7**). These results indicate that alleviation of ZnO NPs-induced oxidative stress by BR was attributed to BR-mediated reduction in Zn content in shoot and root of tomato seedlings under ZnO NPs stress.

DISCUSSION

Despite the discrepancy in research findings relating to NPs-induced phytotoxicity, it is now well evident that large scale use of metal-based NPs (MNPs) could appear as a serious threat to crop production and food safety (Chichiriccò and Poma, 2015; Van Aken, 2015). Development of approaches that can alleviate MNPs-induced phytotoxicity may ensure sustainable crop production in the MNPs-polluted marginal lands. In the current study, for the first time, we showed that ZnO NPs-induced phytotoxicity could be alleviated by exogenous application of BR at optimal concentration (5 nM). BR efficiently ameliorated ZnO NPs-induced oxidative stress by promoting antioxidant enzyme activities, redox homeostasis and related gene expression (**Figures 2–6**). In addition, BR decreased Zn content in both shoot and root of young tomato seedling under ZnO NPs stress (**Figure 7**). This study provides promising data that support beneficial role of BR in enhancing plant tolerance to ZnO NPs stress and ensuring food safety.

Phytotoxicity of MNPs is not only due to their small size, large surface area and intrinsic reactivity that allow them to

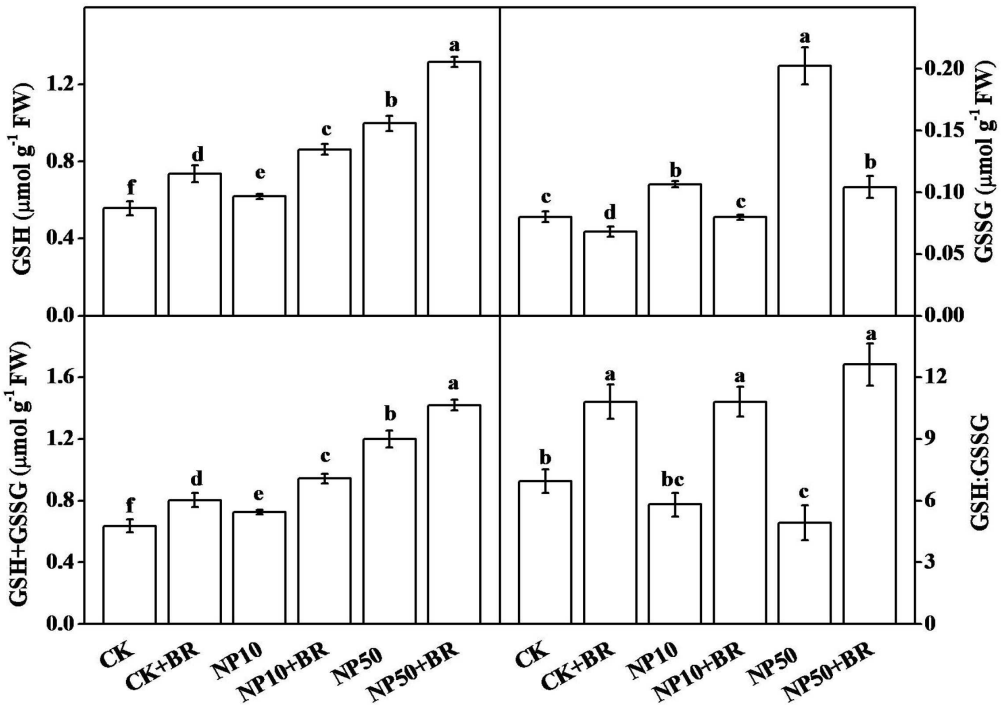

FIGURE 5 | Effects of zinc oxide nanoparticules (ZnO NPs) and brassinosteroid on glutathione content and redox homeostasis in roots of tomato seedlings. Tomato seeds after sprouting were placed on ½ MS medium containing 5 nM 24-epibrassinolide (BR) and/or 10 and 50 mg/L ZnO NPs (NP10 and NP50, respectively) and cultured for 15 days before the analysis of glutathione content. The data shown here are the averages of four replicates, with the standard errors indicated by the vertical bars. The means denoted by the same letter did not significantly differ at a $P < 0.05$, according to Tukey's test. GSH, reduced glutathione; GSSG, oxidized glutathione; GSH+GSSG, total glutathione; GSH:GSSG, ratio between reduced and oxidized glutathione.

interact with biological macromolecules (protein and nucleic acid), but also associated with the metal constituents. Prior research has shown that MNPs dissolve quickly releasing metal ions that induce production of ROS such as H_2O_2 and $^{\bullet}OH$ that are capable to damage vital biomolecules including protein, lipids and DNA (Van Aken, 2015). It is anticipated that ZnO NPs firstly dissolve and then penetrate the cells in the form of Zn^{2+} (Chichiriccò and Poma, 2015). Once they penetrate into the plant, deleterious effects may be produced. In the present study, ZnO NPs exerted a dose-dependent inhibitory effect on tomato growth and biomass accumulation, which was closely associated with accumulation of Zn in plant tissue with increasing concentration of ZnO NPs in culture media (**Figure 1**). ZnO NPs have been shown to reduce root length in corn and cucumber (Zhang et al., 2015), which supports our current observation. It is quite plausible that ZnO NPs inhibit seedling growth concomitantly by directly interacting with biomolecules and also inducing ROS production (Chichiriccò and Poma, 2015). Excessive production of ROS eventually causes an oxidative stress that may lead to cell death (Baxter et al., 2014). To confirm occurrence of oxidative stress upon ZnO NPs treatment, we visualized H_2O_2 accumulation and biochemically quantified concentration of H_2O_2 and MDA, an important marker of lipid peroxidation (**Figure 3**). Our data clearly indicate that ZnO NPs-induced growth inhibition was associated with excessive levels H_2O_2 and MDA. These results are well in accordance with prior reports that showed that MNPs caused plant growth inhibition by inducing oxidative stress in a range of plant species (Dietz and Herth, 2011; Chichiriccò and Poma, 2015).

Besides regulating plant growth and development, phytohormone BR plays a critical role in controlling plant stress responses (Divi et al., 2016). These properties have established BR as a promising plant hormone that could stabilize crop yield under various abiotic stresses such as drought, salinity, extreme temperatures, heavy metals, and organic pollutant stress (Ahammed et al., 2014; Divi et al., 2016). We previously showed that BRs confer Cd tolerance by reducing H_2O_2 accumulation and activating antioxidant potential in tomato (Ahammed et al., 2013). Recently, Ramakrishna and Rao (2015) reported that foliar application of BR mitigates ZnO-induced oxidative stress in radish. In conformity with earlier reports, here we observed that addition of 5 nM BR in ½ MS media remarkably increased tomato seedling growth and biomass accumulation under 10 and 50 mg/L ZnO NPs stress (**Figure 2**). This response of BR was highly dose-specific as we noticed an inhibitory effect of BR on seedling growth when applied at 10 nM concentration (data not shown). It is worth mentioning that BRs-regulated plant stress tolerance in a highly dose-dependent manner that greatly differs across the plant species as well as growth stage and tissue type (Ahammed et al., 2014). In tomato, exogenous BR application or endogenous BR level upregulation by overexpression of BR biosynthetic gene *DWARF* results

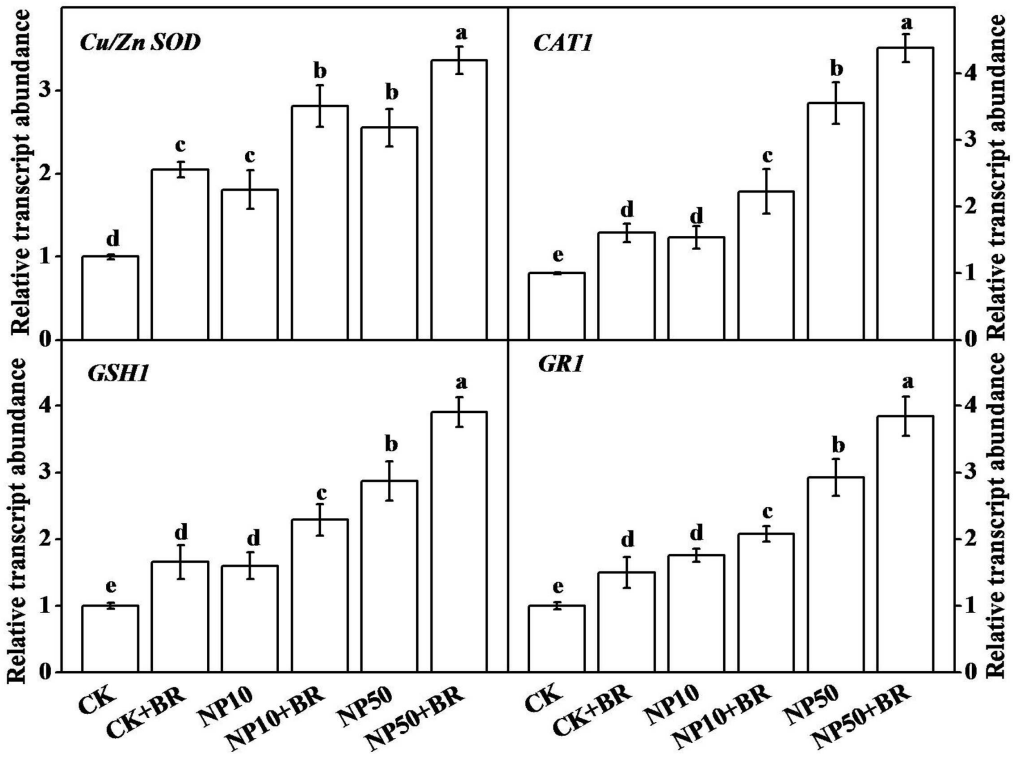

FIGURE 6 | Expression of antioxidant genes in tomato roots as influence by brassinosteroid and zinc oxide nanoparticles (ZnO NPs). The expression of genes was analyzed by qRT-PCR after 15 days treatment using gene-specific primer pairs. The data shown here are the averages of four replicates, with the standard errors indicated by the vertical bars. The means denoted by the same letter did not significantly differ at a $P < 0.05$, according to Tukey's test. BR, 5 nM 24-epibrassinolide; CK, control; NP10, 10 mg/L ZnO NPs; NP50, 50 mg/L ZnO NPs.

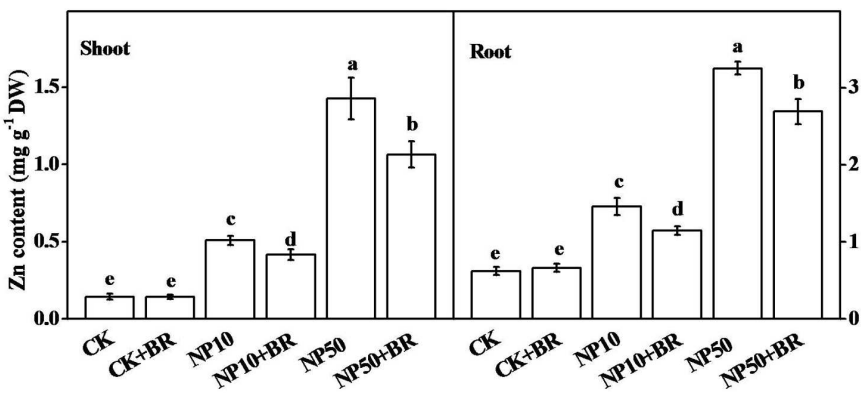

FIGURE 7 | Interactive effect of zinc oxide nanoparticles (ZnO NPs) and brassinosteroid on accumulation of Zinc in tomato seedlings. Tomato seeds after sprouting were placed on ½ MS medium containing 5 nM 24-epibrassinolide (BR) and/or 10 and 50 mg/L ZnO NPs (NP10 and NP50, respectively) and cultured for 15 days before the analysis of Zn content. The data shown here are the averages of four replicates, with the standard errors indicated by the vertical bars. The means denoted by the same letter did not significantly differ at a $P < 0.05$, according to Tukey's test.

in improved plant growth (Li et al., 2015). BR stimulates cell division, elongation and development that are closely associated with BR-promoted plant growth (Ahammed et al., 2014; Divi et al., 2016).

Reactive oxygen species play a dual role in plant system; on one hand they serve as a critical signaling molecule and on the other hand they cause toxicity when accumulated at high level especially under stress (Baxter et al., 2014). Our prior study showed that H_2O_2, a typical ROS, is intricately involved in BR-mediated stress response (Xia et al., 2009). BR triggers transient accumulation of NADPH-based H_2O_2 which in turn stimulates antioxidant potential by inducing transcript of antioxidant

biosynthetic genes (Cui et al., 2011; Nie et al., 2013; Divi et al., 2016). BR-promoted activities of antioxidant enzymes eventually increase ROS scavenging and thus minimizing oxidative stress under stress (Cui et al., 2011). In the current study, BR treatment significantly reduced accumulation of harmful level of H_2O_2 under ZnO NPs stress (**Figure 3A**). In addition to reduction in H_2O_2 content, BR-mediated decrease in MDA content revealed that BR efficiently alleviated ZnO NPs-induced oxidative stress in tomato seedlings (**Figure 3C**). This observation was further confirmed by analysis of antioxidant enzyme activities. Supplementation of BR remarkably increased activities of SOD, CAT, APX, and GR under ZnO NPs stress. In plant cells, SOD can rapidly convert $O_2^{\bullet -}$ to H_2O_2. Once levels of H_2O_2 are high due to catalytic activity of SOD or stress induction, some other antioxidative pathways may be activated to diminish excessive H_2O_2. CAT is a common antioxidant enzyme that converts H_2O_2 to H_2O and O_2. Besides, APX can also convert H_2O_2 to H_2O involving ascorbate-glutathione cycle. In addition, GSH is a key antioxidant molecule and can directly decompose H_2O_2 by producing oxidant glutathione (GSSG), which is then reduced to GSH by GR (Ma et al., 2015). Our results are in conformity with many earlier reports that showed that application of BR conferred stress tolerance by inducing antioxidant enzyme activities in different plant species (Ramakrishna and Rao, 2012, 2015, p. 29; Ahammed et al., 2013, 2014; Vardhini and Anjum, 2015).

Maintenance of cellular redox homeostasis is critical for normal metabolic processes in plants (Baxter et al., 2014). Under MNPs stress, redox balance is disrupted due to over accumulation of ROS (Chichiriccò and Poma, 2015; Van Aken, 2015). In the current study, GSH:GSSG ratio was significantly decreased under 50 mg/L ZnO NPs stress, indicating an oxidized redox state which is considered harmful for plants. Ag NPs stress also reduced plant biomass but increased accumulation of oxidized glutathione (GSSG, indicative of oxidative stress) in *Triticum aestivum* (Van Aken, 2015). Treatment with BR decreased GSSG content under ZnO NPs stress, which helped young tomato seedlings to maintain a high GSH:GSSG ratio that was almost equivalent to non-stressed seedlings (**Figure 5**). These results are in agreement with earlier reports that showed that BRs could maintain redox homeostasis under Zn and Cd stress in radish and tomato, respectively (Ramakrishna and Rao, 2012; Ahammed et al., 2013).

Brassinosteroid enhances plant tolerance to broad-range stresses, while such enhancement in stress tolerance is correlated with higher expression of genes that are considered as stress markers (Divi et al., 2016). Transcription of antioxidant related genes are generally upregulated under stress (Baxter et al., 2014). To study the molecular bases of the toxicity of ZnO NPs and alleviatory role of BR, we studied expression of antioxidant related genes by using qRT-PCR. In this study, transcripts of *Cu/Zn SOD, GSH1, CAT1,* and *GR1* were induced by ZnO NPs treatment and BR co-application further upregulated their transcript abundance in roots of tomato seedlings. This observation is in line with previous reports, which showed that BR could upregulate expression of various antioxidant related genes in rice and tomato under Cr and Cd stress, respectively (Ahammed et al., 2013; Sharma et al., 2016).

Given that plants are vital components of the food chain, its interaction with NPs essentially have major implications in understanding of environment and public health (Van Aken, 2015). Concerning the connection between BR and food safety, a number of studies have shown that BR could reduce heavy metal accumulation in plants (Ahammed et al., 2013, 2014; Ramakrishna and Rao, 2015; Sharma et al., 2016). In conformity with earlier reports, here we found that BR treatment caused a significant reduction in Zn accumulation under ZnO NPs stress in tomato seedling (**Figure 7**). This implies that BR could also reduce accumulation of metals from MNPs, and thus suggesting a potential implication of BR in ensuring food safety in MNPs-polluted areas. In a recent study, (Chen et al., 2015) showed that NO could alleviate ZnO NPs-induced phytotoxicity by reducing Zn accumulation in rice seedlings. NO is an important signaling molecule that enhances plant tolerance to various environmental stresses. In our earlier study, we found that NO is involved in BR-induced oxidative stress tolerance in cucumber (Cui et al., 2011). Therefore, BR-induced alleviation of ZnO NPs phytotoxicity might also be mediated by regulation of NO (Cui et al., 2011; Chen et al., 2015).

Taken together, this study showed that ZnO NPs induced oxidative stress by increasing accumulation of Zn in plant tissue, which in turn reduced plant growth and biomass accumulation possibly by affecting vital cellular processes. In contrast, treatment with BR alleviated ZnO NPs-induced growth inhibition and oxidative stress by improving antioxidant potential and redox homeostasis. The alleviation of ZnO NPs-induced oxidative stress by BR, was closely associated with upregulation of transcripts of antioxidant genes. BR-induced reduction in Zn content under ZnO NPs stress, provides new prospect for stabilizing crop yield and quality in MNPs-contaminated land. To our knowledge, this study provides the first evidence that BR could alleviate ZnO NPs-induced phytotoxicity in tomato. However, further study involving advanced genetic and molecular tools may help to better understand the detailed molecular mechanisms of BR-induced ZnO NPs tolerance in plants.

AUTHOR CONTRIBUTIONS

JZ, ML, GA, and JY planned and designed the research. ML, CL, XB, CH, and HY performed experiments. JZ, ML, and GA analyzed data. JZ, ML, and GA wrote the manuscript.

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REVIEW
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Tuning of Redox Regulatory Mechanisms, Reactive Oxygen Species and Redox Homeostasis under Salinity Stress

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Soil salinity is a crucial environmental constraint which limits biomass production at many sites on a global scale. Saline growth conditions cause osmotic and ionic imbalances, oxidative stress and perturb metabolism, e.g., the photosynthetic electron flow. The plant ability to tolerate salinity is determined by multiple biochemical and physiological mechanisms protecting cell functions, in particular by regulating proper water relations and maintaining ion homeostasis. Redox homeostasis is a fundamental cell property. Its regulation includes control of reactive oxygen species (ROS) generation, sensing deviation from and readjustment of the cellular redox state. All these redox related functions have been recognized as decisive factors in salinity acclimation and adaptation. This review focuses on the core response of plants to overcome the challenges of salinity stress through regulation of ROS generation and detoxification systems and to maintain redox homeostasis. Emphasis is given to the role of NADH oxidase (RBOH), alternative oxidase (AOX), the plastid terminal oxidase (PTOX) and the malate valve with the malate dehydrogenase isoforms under salt stress. Overwhelming evidence assigns an essential auxiliary function of ROS and redox homeostasis to salinity acclimation of plants.

Keywords: alternative oxidase, antioxidant enzymes, hydrogen peroxide, NADPH oxidase, salinity stress

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INTRODUCTION

Soil salinity is a major environmental stress that strongly impairs crop productivity and harvest quality in the world (Horie and Schroeder, 2004). Significant areas of the cultivated land in more than 100 countries are affected by salinity (Rengasamy, 2006). The quality of approximately 20% of the world's cultivated area and about 50% of the world's irrigated lands is affected by salinization (Sairam and Tyagi, 2004). Hence, soil salinity poses a serious threat to crop yield and future food production. Plant responses and tolerance mechanisms to salt stress are a major topic of plant research (Munns and Tester, 2008). In general, high salt concentrations induce ionic imbalances, osmotic stress and oxidative damage (Zhu, 2001). Glycophytic plants under salt stress conditions exhibit slow growth, wilting and eventually death (Parida et al., 2004). To survive under stress condition, plants respond and adapt with complex mechanisms that include developmental, morphological, physiological and biochemical strategies (Taji et al., 2004; Acosta-Motos et al., 2015) addressing ion homeostasis, osmolyte biosynthesis, compartmentation of toxic ions, and reactive oxygen species (ROS) scavenging systems (Stepien and Klobus, 2005; Flowers and Colmer, 2008). Many genes involved in membrane transport, signal transduction, redox reactions

and other processes have been identified and characterized (Inan et al., 2004; Zhang et al., 2008). However, the quantitative contribution of the various molecular mechanisms, their qualitative interactions and the integrated functional network underlying plant tolerance to salt stress remain to be determined.

Cell metabolism generates reactive oxygen species (ROS) at low rates as normal side product. Salinity stress often enhances the generation of reactive oxygen species (ROS). This may lead to metabolic disorders, cellular damage, and premature senescence or necrosis (Møller et al., 2007; Jaleel et al., 2009; Miller et al., 2010; Habib et al., 2016). Excessively accumulating ROS may react with suitable targets such as nucleic acids, proteins, lipids and chlorophyll. The main ROS include non-radical molecules like singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2), as well as free radicals such as superoxide ($O_2^{\bullet-}$) and hydroxyl radicals ($^\bullet OH$) (Azevedo Neto et al., 2008). Besides their harmful effects ROS act as signaling molecules that regulate plant development, biotic and abiotic stress responses (Mittler et al., 2004). Recent research and considerations have focused on ROS metabolism (Noctor et al., 2014), sensory and signaling networks (Dietz, 2008; Miller et al., 2010; Suzuki et al., 2012; Baxter et al., 2014), as well as the cross-talk with other signaling pathways (Suzuki et al., 2012; Noctor et al., 2014).

The seemingly negative consequences of excess ROS accumulation like lipid peroxidation, oxidation of proteins, damage of nucleic acids, enzyme inhibition, and activation of programmed cell death (PCD) are also linked to signaling since the reaction products transmit information to downstream events (**Figure 1**; Mishra et al., 2011; Srivastava and Dubey, 2011). The steady state ROS levels depend on the rates of generation and decomposition (**Figure 1**). Three levels of specificity need to be considered at the level of ROS, their chemical reactivity, as well as their temporal and spatial accumulation pattern. A network of low molecular mass antioxidants and antioxidant enzymes, redox input elements, redox transmitters, redox target proteins and redox sensors orchestrate the readjustment of redox homeostasis and redox-dependent response (Azevedo Neto et al., 2008; Dietz, 2008; Gill and Tuteja, 2010). To understand the redox and ROS balance under salinity we have to consider (i) the ROS generator systems, (ii) the antioxidant defense system, and (iii) the redox regulatory network.

The best studied component is the antioxidant system which includes gene families encoding superoxide dismutases (SOD), catalases (CAT), guaiacol peroxidases (POX), the ascorbate-glutathione (ASC-GSH) cycle enzymes [ascorbate peroxidases (APX), monodehydroascorbate reductases (MDHAR), dehydroascorbate reductases (DHAR), glutathione reductases (GR)], glutathione peroxidases (GPX), peroxiredoxins (PRX) and glutathione S-transferases (GST) (Mittler et al., 2004; Azevedo Neto et al., 2008; Munns and Tester, 2008). These antioxidant enzymes are targeted to specific subcellular compartments and reveal stress-specific modulation of their expression as compiled e.g., by Mittler et al. (2004) for *A. thaliana*.

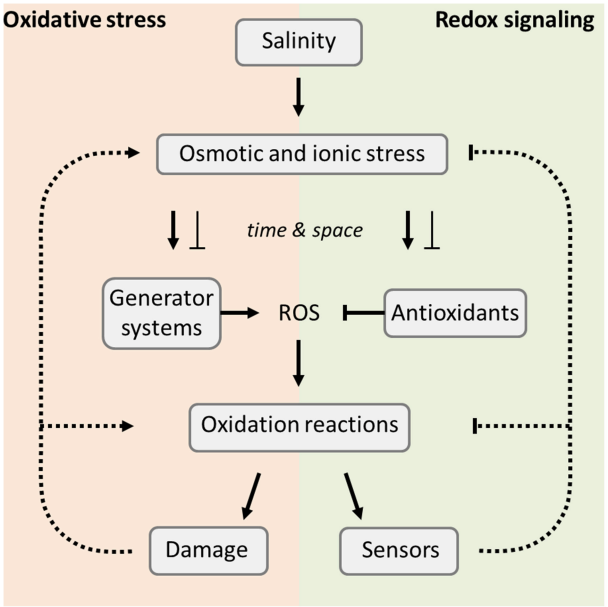

FIGURE 1 | Overview of salinity-induced stress effects. The primary stress effects are osmotic and ionic imbalances which affect the ROS generator and antioxidant systems. This effect may be positive or negative as indicated and discussed. Their relative activities determine the ROS levels, as well as the subsequent redox reactions which are used in signaling **(right hand side)** or, if excessive, cause oxidative damage which enhances the stress effects **(left hand side)**.

Intensive research on salinity has addressed the ROS generator systems which in a regulated or conditional, but partly less targeted manner control the release of ROS. Metabolic pathways like photorespiration and membrane-associated enzymes determine the rate of ROS generation in plant cells; the NADPH oxidase (respiratory burst oxidase homolog: RBOH) (Keller et al., 1998), mitochondrial alternative oxidase (AOX) (Considine et al., 2002) and plastid terminal oxidase (PTOX) (Stepien and Johnson, 2009). Additional layers of defense and regulation modulate the redox state of the cell and control the subcellular redox balance. Malic acid and oxaloacetic acid represent a redox pair of metabolites linked by malate dehydrogenases (MDH), e.g., the NADP-MDH in the chloroplast. Exchange of malic acid and oxaloacetic acid by membrane transport among compartments allows for indirect transfer of reducing equivalents. This process is known as malate valve (Scheibe et al., 2005). The tightly regulated plastid NADP-MDH controls the export of excess reducing power from the photosynthesizing chloroplast and thereby relieves overreduction of the photosynthetic electron transport chain and thus generation of ROS.

Abbreviations: AOX, alternative oxidase; APX, ascorbate peroxidase; CAT, catalase; CEF, cyclic electron flow; DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GST, glutathione-S-transferase; MDH, malate dehydrogenase, NADP- or NAD-dependent; MDHAR, monodehydroascorbate reductase; PET, photosynthetic electron transport; PRX, peroxiredoxin; POD, guaiacol heme peroxidase; PTOX, plastid terminal oxidase; RBOH, respiratory burst oxidase homologue; RET, respiratory electron transport; ROS, reactive oxygen species; SOD, superoxide dismutase; TRX, thioredoxin.

The significance of these enzymes and mechanisms in salt tolerance is still a matter of controversy. Often, regulatory patterns are difficult to interpret; e.g., upregulation of antioxidant enzymes may represent the response to manifested oxidative stress. On the other hand, upregulation of antioxidant enzymes may be considered as proactive acclimation response which results in lower ROS levels and higher tolerance to oxidative stress. Consequentially high antioxidant enzyme activities have been associated with salt tolerance as well as salt sensitivity. With that said, this review aims to provide a critical update on redox regulation, oxidative stress and their implications in salt stress acclimation and damage development.

GENERATION OF ROS UNDER SALINITY STRESS IN PLANT

Salt stress interferes with carbon metabolism and thereby fosters ROS generation. Stomatal conductance decreases during salt stress and lowers transpiration. The stomatal movement is linked to ion redistribution, alkalization and ABA accumulation (Geilfus et al., 2015). Restricted gas exchange limits CO_2 uptake, lowers intercellular CO_2 concentration and CO_2 availability for the Calvin cycle. As a consequences the pool of oxidized $NADP^+$ (as final electron acceptor at PSI) is depleted and electrons are transferred to O_2 to generate $O_2^{\bullet-}$ (Mehler, 1951). Following non-enzymatic or enzymatic dismutation by superoxide dismutase (SOD), H_2O_2 can be converted to the extremely reactive hydroxyl radicals ($^{\bullet}OH$) in the Fenton-/Haber-Weiss-reaction. Insufficient energy dissipation in photosynthesis causes formation of $^1O_2$ from triplet chlorophyll (Chl) especially in the reaction center of photosystem II (Krieger-Liszkay, 2005). The decrease in the CO_2/O_2-ratio in the mesophyll enhances photorespiration in C3-plants and stimulates H_2O_2 generation in the peroxisome (Wingler et al., 2000; Ghannoum, 2009). Photorespiration accounts for over 70% of the H_2O_2 production under osmotic stress (Noctor et al., 2002). Early studies on respiratory electron transport (RET) reported increased rates of electron transfer to O_2 and thus of respiratory O_2 consumption under salt stress (Fry et al., 1986; Moser et al., 1991). $O_2^{\bullet-}$ is generated when RET is overreduced. The generated $O_2^{\bullet-}$ is dismutated to H_2O_2 which is subsequently reduced to water by catalases, class I peroxidases (APXs), class III peroxidases (POXs) and thiol peroxidases. Salinity stress activates the cell membrane-bound RBOH (Rejeb et al., 2015a; Tsai et al., 2005) and the apoplastic diamine oxidase (Waie and Rajam, 2003). Both mechanisms contribute to the generation of ROS in the apoplastic space. In other cases RBOH activity was inhibited under salt stress (Rodríguez et al., 2009). In salt-stressed maize leaves (150 mM NaCl in hydroponics), levels of apoplastic spermidine and spermine increased several times and the apoplastic polyamine oxidase allowed for converting the polyamines to 1,3-diaminopropane and H_2O_2 (Rodríguez et al., 2009). This mechanism enables cell wall loosening by generation of $^{\bullet}OH$ and sustains leaf blade growth even if RBOH is inhibited (Rodríguez et al., 2009). Other ROS-generating enzymes include oxalate oxidase and amine oxidase. In context of salinity much work has focused on RBOH-like enzymes, the major enzymatic route of ROS synthesis in plant cells (Sharma et al., 2012), which will be discussed below in more detail.

Under salinity stress each type of organelle employs different mechanisms of ROS production. The subcellular and cellular sites of ROS production decisively determine their signaling action. Thus, ROS are produced in particular in the PET of chloroplasts, the RET in mitochondria, various oxidases in peroxisome and the NADPH oxidase (RBOH) in the plasma membrane (**Figure 2**). In addition reactions in the endomembrane system and the apoplast/cell wall also contribute to ROS generation (**Table 1**). Localized production in specific micro-compartments and the buffering action of multiple antioxidant mechanisms fine-tune the concentrations of ROS at particular sites of the cell under salt stress. Such mechanisms could delimit the response to localized "hot-spots."

SCAVENGING OF ROS UNDER SALINITY STRESS IN PLANT

Superoxide dismutase (SOD) belongs to the group of metalloenzymes and functions as an important enzyme in the first line of antioxidant defense. MnSOD, Cu/ZnSOD and FeSOD dismutate $O_2^{\bullet-}$ into H_2O_2 and O_2 (Rios-Gonzalez et al., 2002; Tuna et al., 2008). Elevated SOD activity often appears to enhance plant tolerance to oxidative stress (Gupta et al., 1993). $^1O_2$ can be quenched by β-carotene or α-tocopherol, but also can react with the D1 protein of photosystem II as a sensitive protein target (Krieger-Liszkay, 2005). Among the antioxidant enzymes, catalase (CAT) was discovered first and dismutates two molecules of H_2O_2 into water and oxygen. Peroxidases are classified as heme or thiol (or selenol) peroxidases and reduce H_2O_2 at the expense of an alternative electron donor (Dietz, 2016). Thiol peroxidases use thiol electron donors such as thioredoxin (TRX), glutaredoxin (GRX), glutathione or in rare cases ascorbate to convert H_2O_2 to H_2O. Ascorbate peroxidase (APX) catalyzes the primary step in the classical water-water cycle (Asada, 1992) where DHAR and GR, a flavoenzyme which contains an essential disulfide group, catalyze the regeneration of ascorbate from dehydroascorbate using glutathione and finally NADPH as reductants. The regeneration of ascorbate from monodehydroascorbate is also carried out by MDHAR using NADH as reducing power. GPX, some GST and PRX reduce H_2O_2 and organic hydroperoxides through ascorbate-independent thiol-mediated pathways using nucleophiles such as GSH, thioredoxin (TRX) or glutaredoxins (GRX) (Asada, 1992; Dietz et al., 2006; Noctor et al., 2014). Transcript levels of TRX, Mn-SOD, AOX, and PRXIIF increased under short-term salinity conditions (Marti et al., 2011). The mitochondrial PsTRXo1 increases in pea leaves in response to long-term salinity (Marti et al., 2011). PsTRXo1 and PRXIIF provide the cell with a mechanism to protect mitochondria from oxidative stress together with Mn-SOD and AOX. Mitochondrial TRX-o1, PRXIIF and sulfiredoxins participate in the establishment of salt tolerance through a fine-regulated mechanism involving the post-translational modifications of *S*-glutathionylation and *S*-nitrosylation (Lázaro et al., 2013).

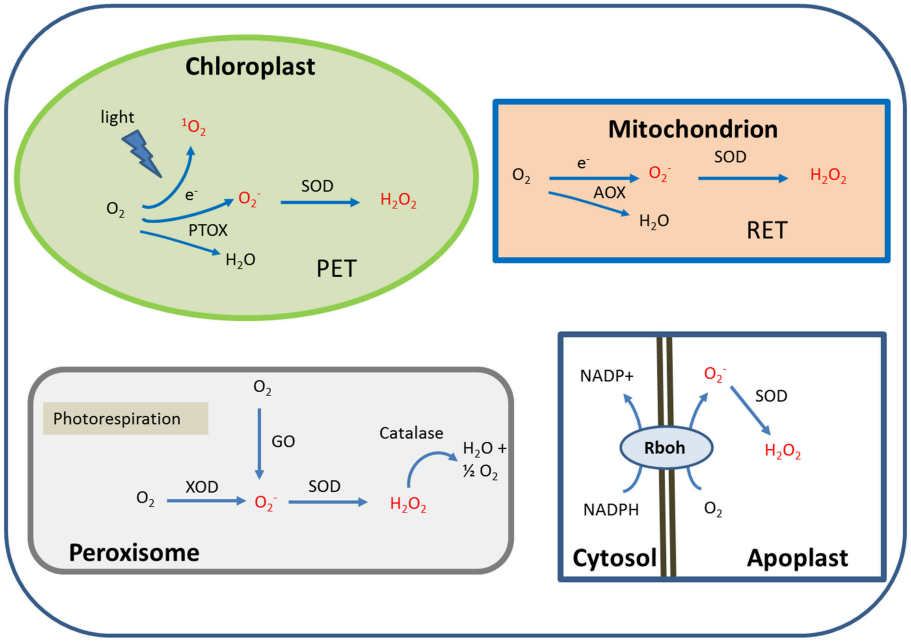

FIGURE 2 | Simplified scheme illustrating the predominant ROS generation sites in plant cell under salinity stress. These sites are photosynthesis in chloroplasts, respiration in mitochondria, photorespiration in peroxisomes and NADH oxidation at the plasma membrane. AOX, alternative oxidase; GO, glycolate oxidase; PET, photosynthetic electron transport; PTOX, plastid terminal oxidase; RBOH, respiratory burst oxidase homolog; RET, respiratory electron transport; SOD, superoxide dismutase; XOD, xanthine oxidase.

TABLE 1 | Generation sites and origin of reactive oxygen species (ROS) in plants.

Location	Key sources of ROS in cell	References
Chloroplast	PET (PSI, PQ, and PSII) Chlorophyll pigments	Elstner, 1991; Cleland and Grace, 1999
Mitochondrion	Complexes of RET Enzymes, e.g., aconitase, 1-galactono-γ lactone dehydrogenase (GAL)	Andreyev et al., 2005; Rasmusson et al., 2008
Peroxisome	Glycolate oxidase (GO), fatty acid β-oxidation, flavine oxidases, xanthine oxidase (XOD), peroxisomal NADPH: cytochrome P450 reductase and ETC composed of a flavoprotein, NADH and Cyt b	López-Huertas et al., 1999; Baker and Graham, 2002
Plasma membrane	NADPH oxidase and menadione (quinone reductase)	Heyno et al., 2011
Apoplast	Cell wall-associated oxalate oxidase (germine) and amine oxidase-like enzymes	Wojtaszek, 1997; Cona et al., 2006
Cell wall	Cell-wall-associated peroxidase in the presence of NADH and diamine oxidases	Gross, 1977; Martinez et al., 1998
Endoplasmatic reticulum	NAD(P)H-dependent electron transport involving Cyt P450	Mittler, 2002

The link between salt tolerance and increased activities of antioxidant enzymes has frequently been established (Zeng et al., 2003; Liu et al., 2011; **Table 2**). Stepien and Klobus (2005) compared oxidative stress in salt-stressed wheat and maize by assessing lipid peroxidation and activities of antioxidant enzymes. Maize suffered less oxidative stress than wheat. The constitutive and salt stress-inducible activities of SOD, APX and GR were higher in maize than in wheat. The authors suggested that the higher tolerance of maize is based on two mechanisms, namely lower ROS production in C4-photosynthesis and higher activities of the antioxidant enzymes. Photorespiration is strongly induced by salt stress in C3 but not in C4 and CAM plant (Cushman and Bohnert, 1997). *Flaveria*, *Alternanthera*, *Parthenium*, *Panicum* and *Moricandia* species are some species with C3-C4 intermediate photosynthesis which are able to efficiently recycle photorespired CO_2 which lowers the rate of photorespiration (Monson et al., 1984; Devi and Raghavendra, 1993). C3 species examined had high intrinsic levels of photorespiration whereas the C3-C4 intermediate species (primarily by refixing photorespired CO_2), C4-like and C4 species (via selective localization of ribulose-1,5-bisphosphate carboxylase in bundle sheath cells and operation of a CO_2

TABLE 2 | Antioxidant enzymes reported to be regulated in plants under salinity stress.

Antioxidant enzymes*	Plant species	References
SOD, **CAT**, **GPX**, **APX**, **GR**, **MDHAR, DHAR**	*Oryza sativa*	Mishra et al., 2013
CAT, **SOD, GR**	*Olea europaea*	Valderrama et al., 2006
GPX	*Oryza sativa*	Mittal and Dubey, 1991
APX, MDHAR, DHAR, GR	*Oryza sativa*	Hossain et al., 2013
SOD	*Triticum aestivum*	Borzouei et al., 2012
	Oryza sativa, Avicennia marina	Prashanth et al., 2008
	Nicotiana tabacum	Van Camp et al., 1996
	Oryza sativa	Tanaka et al., 1999
	Cakile maritime	Ellouzi et al., 2011
GR, **SOD**, **POX, CAT**	*Helianthus annuus, Zea mays*	Rios-Gonzalez et al., 2002
SOD, **APX, DHAR**	*Nicotiana tabacum*	Lee et al., 2007
APX, GR	*Oryza sativa*	Tsai et al., 2005
SOD, APX, GR	*Triticum aestivum*	Stepien and Klobus, 2005
SOD, **APX, GR**	*Zea mays*	Stepien and Klobus, 2005
CAT, **APX, GR**	*Arabidopsis thaliana*	Rejeb et al., 2015a
APX	*Nicotiana tabacum*	Badawi et al., 2004; Li et al., 2009; Sun et al., 2010
	Pisum sativum, Lycopersicon esculentum	Wang et al., 2005
	Hordeum vulgare	Shi et al., 2001
	Arabidopsis thaliana	Lu et al., 2007
	Ipomoea batatas	Lin and Pu, 2010
DHAR, GR, MDHAR, APX, SOD	*Pisum sativum*	Hernández et al., 2001
CAT, **POX**, **APX, GR**, SOD	*Echinochloa crusgalli*	Abogadallah et al., 2009
MDHAR	*Nicotiana tabacum*	Eltayeb et al., 2007
DHAR	*Lotus japonicas*	Rubio et al., 2009
GR	*Nicotiana tabacum)*	Aono et al., 1993
	Populus species	Foyer et al., 1995
SOD, **CAT**, **APX, MDHAR**	*Lycopersicon esculentum*	Shalata et al., 2001
SOD, **CAT, APX**	*Gossypium hirsutum*	Luo et al., 2013
POX, CAT, SOD	*Vigna unguiculata*	Cavalcanti et al., 2004
NOX/RBOH	*Arabidopsis thaliana*	Sakamoto et al., 2008; Ma et al., 2012
NOX/RBOH	*Glycine max*	Song et al., 2012
NOX/RBOH	*Brassica juncea*	Srivastava et al., 2015
NOX/RBOH	*Sesuvium portulacastrum*	Srivastava et al., 2015
RBOHD/F	*Arabidopsis thaliana*	Ma et al., 2012
RBOHF	*Arabidopsis thaliana*	Jiang et al., 2012
AOX	*Citrus sinensis (cvs. Carvalhal tangor)*	Ferreira et al., 2008
	Glycine max	Hilal et al., 1998
	Arabidopsis thaliana	Smith et al., 2009; Wang et al., 2010
	Hordeum vulgare	Jolivet et al., 1990
OsAOX1a	*Oryza sativa*	Li et al., 2013
OsAOX1b	*Oryza sativa*	Li et al., 2013
OsAOX1c	*Oryza sativa*	Li et al., 2013
NADH-MDH	*Oryza sativa*	Kumar et al., 2000
	Mesembryanthemum crystallinum	Cushman, 1993; Gawronska et al., 2013
	Arabidopsis thaliana	Hebbelmann et al., 2012
PTOX	*Thellungiella halophila*	Stepien and Johnson, 2009
PTOX	*Arabidopsis thaliana*	Josse et al., 2003; Stepien and Johnson, 2009
	Oryza sativa	Kong et al., 2003
	Haematococcus pluvialis	Wang et al., 2009
	Thellungiella salsuginea	Wiciarza et al., 2015

**Bold: upregulated under salt stress, underlined: down-regulated, normal: unchanged.*

pump via the C4 pathway) maintained low apparent rates of photorespiration (Dai et al., 1996). In another study, Rios-Gonzalez et al. (2002) reported higher activities of GR, SOD, POD and CAT in sunflower leaves than in maize under salt stress. C4-like *Flaveria brownie* and C4 *Flaveria bidentis* are able to prevent oxidative damage by stress by increased enzymatic and non-enzymatic antioxidants, as compared to C3 (*Flaveria robusta*) and C3–C4 intermediate (*Flaveria anomala*) (Uzilday et al., 2014). Higher water use efficiency of C4 plants should support growth on saline soil. However, it appears too early to generalize on a possible advantage that C4 plants possibly have since many other traits participate in salinity tolerance a priori.

A higher redox status of antioxidants and the coordinated increase in SOD, CAT, GPX, APX, and GR activities was suggested by Mishra et al. (2013) to serve as the major determinants of salt tolerance in Indica rice seedlings. In this study, the activity of CuZn-SOD, APX, GPX, CAT, MDHAR, DHAR, and GR increased in the salt tolerant cultivar like in salt-sensitive seedlings at moderate salinity of 7 dS m^{-1} NaCl. In contrast, the activity of GPX, CAT, MDHAR, DHAR, and GR decreased with higher salinity of 14 dS m^{-1} NaCl in the sensitive genotype. Cultivars with distinct salt sensitivity allow exploring involved mechanisms. Pusa Basmati-1 is highly salt sensitive, while Pokkali displays moderate salt tolerance. In parallel the activity of the ROS scavenging CAT and the levels of antioxidants like ASA and GSH are increased and concomitantly the membrane damage as judged from lipid peroxidation and H_2O_2 levels are lower in Pokkali compared to Pusa Basmati-1 (Vaidyanathan et al., 2003). Transcript regulation of peroxisomal APX (HvAPX1) correlates with salt stress (Shi et al., 2001). Lin and Pu (2010) studied the involvement of ROS scavenging enzymes in tolerant and sensitive sweet potato under salinity. The activity increase of cAPX, mAPX and chlAPX 24 and 48 h after exposure to 450 mM NaCl was higher in the salt stress-tolerant genotype than in the sensitive ones. Overall induction and maintenance of a strong antioxidant defense frequently correlates with enhanced salt tolerance.

Likewise genetic fortification of antioxidant levels has been shown to enhance salinity tolerance by decreasing the oxidative stress. Transgenic tobacco overexpressing cytosolic AtMDAR1 exhibited up to 2.1-fold higher MDAR activity and 2.2-fold higher levels of reduced AsA than non-transformed control plants and concomitantly the tolerance to salt stress was enhanced (Eltayeb et al., 2007). Likewise, transgenic Arabidopsis over-expressing rice cytosolic OsAPXa/b exhibited increased salt tolerance compared to wild-type (Lu et al., 2007). A similar improvement in salt stress tolerance was observed in transgenic tobacco expressing the AtcAPX gene (Badawi et al., 2004) or the *Solanum lycopersicum* tAPX (SltAPX) (Sun et al., 2010). Transgenic tobacco simultaneously expressing CuZnSOD, APX, and DHAR in the chloroplast tolerated 100 mM NaCI without developing salt-induced injury observed in wild type (Lee et al., 2007). Prashanth et al. (2008) applied salt stress to indica rice var Pusa Basmati-1 overexpressing cytosolic Cu/ZnSOD from the mangrove *Avicennia marina*. The heterologous expression conferred salinity stress tolerance in hydroponics and pot experiments. GhSOD1-, GhAPX1-, and GhCAT1-overexpressing cotton showed higher tolerance to salinity than WT, and the synergistic effects of GhSOD1 and GhCAT1 were suggested to provide a new strategy for enhancing salt stress tolerance (Luo et al., 2013). Overall the results from transgenic approaches are complementary to the correlative evidence at the level of regulation of enzyme activity and of transcripts amounts and support the conclusion that enhanced antioxidant activity fosters salt acclimation. **Figure 3** summarizes the various antioxidant systems in chloroplast, mitochondrion and peroxisome. It also indicates the sites of the safety valves and generator systems which will be discussed next, the alternative oxidase (AOX), the plastid terminal oxidase (PTOX), the respiratory burst oxidase homolog (RBOH) and the malate valve.

ALTERNATIVE OXIDASE (AOX)

Redox and ROS metabolism are tightly linked. Overreduction of electron transport chains enhances ROS production as discussed above. In higher plants, the respiratory electron transport chain (RET) feeds electrons to two terminal oxidases, namely cytochrome c oxidase (complex IV) for ATP synthesis and the cyanide-insensitive AOX for energy dissipation (Rogov et al., 2014). The conditional bifurcation to both pathways maintains the energy balance as well as redox homeostasis in dependence on the cellular energy demand. Drainage of electrons into the AOX pathway increases under stress (Zhang et al., 2016). Earlier the AOX capacity was demonstrated to respond to salinity in plants such as barley (Jolivet et al., 1990), soybean (Hilal et al., 1998) and carrot (Ferreira et al., 2008). Activation of AOX1a occurs via a thiol switch mechanism (Winger et al., 2007) and stimulates alternative respiration, decreases electron pressure and prevents over-reduction of the ubiquinone (UQ) pool which dampens excessive $O_2^{\bullet-}$ and H_2O_2 generation and lowers ROS damage to plant cells under salt stress (Wang et al., 2010; Yoshida et al., 2011). In addition, AOX is involved in defining the threshold for the induction of programmed cell death (PCD) by signaling mechanism (Van Aken et al., 2009) and modulating the release of reactive nitrogen species (RNS). Release of nitric oxide (NO) in mitochondria is induced by accumulating reducing equivalents (Cvetkovska et al., 2014; Igamberdiev et al., 2014). In rice, expression of AOX1A and AOX1B in the nucleus is stimulated during saline conditions through mitochondrial retrograde regulation mediated by oxygen radicals (Li et al., 2013; Voss et al., 2013). Smith et al. (2009) reported AOX activation and stimulation of alternative electron transport in response to salinity stress. This mechanism allowed for suppressing ROS generation and increased the growth rates. Lower Na^+ accumulation in shoots suggests a link between long distance Na^+ transport and tissue ROS levels. This tentative dependency offers a mechanistic framework to exploit AOX and redox homeostasis to improve the acclimation ability to salt stress.

RESPIRATORY BURST OXIDASE HOMOLOGS (RBOH)

Homologs of NADPH oxidase in plants and animals contain cytosolic FAD- and NADPH-binding domains and six

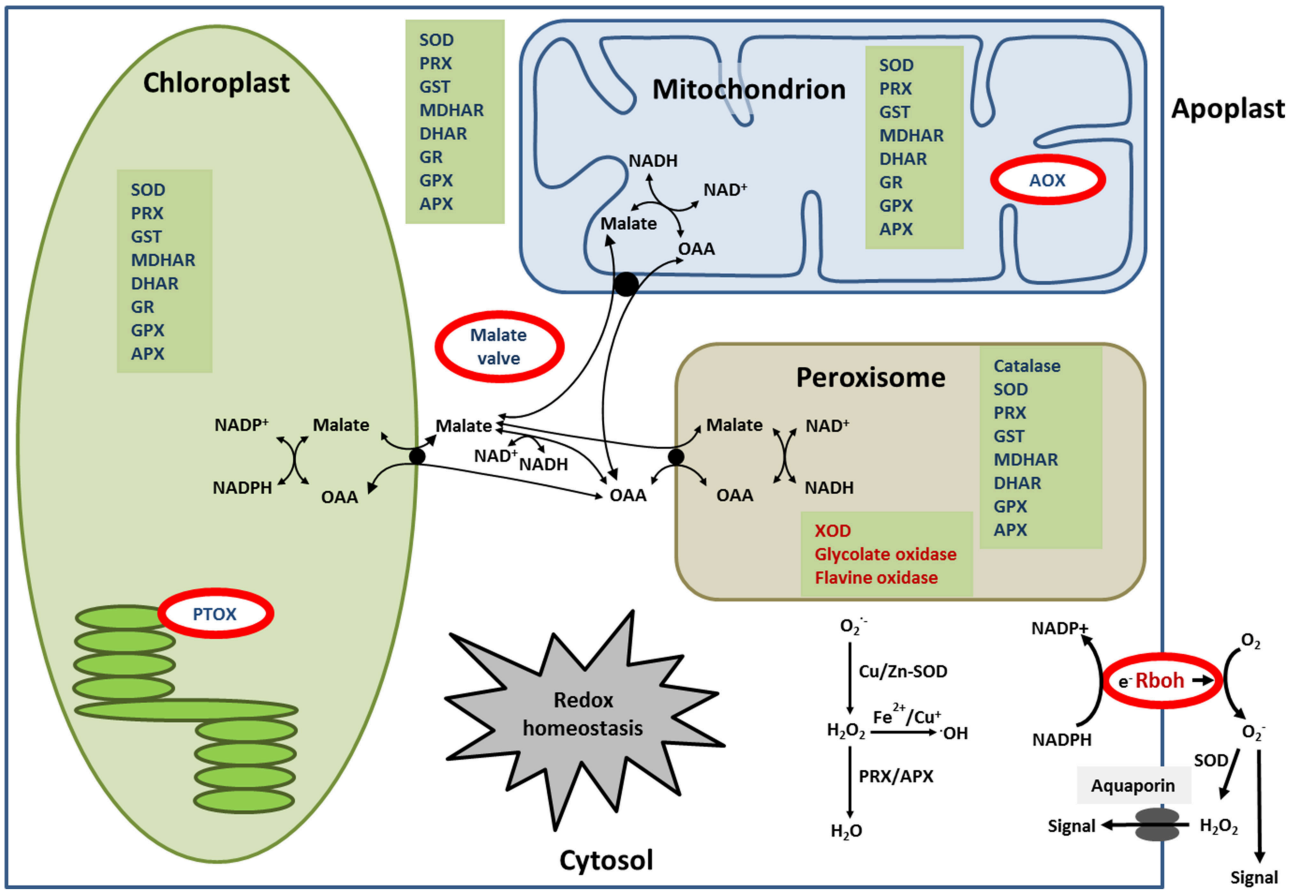

FIGURE 3 | Overview of the antioxidant systems and the malate valve. This figure shows the distribution of the various antioxidant defense systems and the function of the malate valve as mechanism to equilibrate reductive power between organelles.

membrane-spanning helices. Two heme groups participate in the transfer of electrons from cytosolic NADPH or NADH to oxygen on the apoplastic side. This transfer produces $O_2^{\bullet-}$ radicals in the extracellular matrix (Lambeth, 2004; Sagi and Fluhr, 2006). In plants, these enzymes are named RBOH and function in a plethora of processes, such as hyersensitive response to pathogens, abiotic stress tolerance, and local and systemic signaling (Torres et al., 2002; Monshausen et al., 2009). ROS released by RBOH act in several hormone-signaling pathways (Sagi and Fluhr, 2006).

RBOHA activity is induced by salt stress in the root elongation zone in Arabidopsis (Sagi and Fluhr, 2006). In Arabidopsis AtRBOHD and AtRBOHF are expressed in all plant organs and are the main isoforms involved in ROS-dependent regulation of Na^+/K^+ homeostasis under salt stress (Ma et al., 2012). AtRBOHD has also been demonstrated to mediate rapid systemic signaling triggered by multiple abiotic stresses (Miller et al., 2009) and to be required for salt acclimation signaling mediated by heme oxygenase HY1 in Arabidopsis (Xie et al., 2011). RBOHD shows the highest expression among the ten AtRBOH genes in *A. thaliana* (Suzuki et al., 2011) and functions in abscisic acid-dependent stomatal closure, flagellin-induced immune responses, and salt acclimation via ROS production (Torres et al., 2002; Pogány et al., 2009; Xie et al., 2011). Under salt stress RBOHD are clustered in the plasmamembrane and subjected to endocytosis and their activation facilitates the activation of redox signaling pathways and plays an important role in salt acclimation of Arabidopsis (Leshem et al., 2007; Xie et al., 2011; Hao et al., 2014).

Mild salt stress causes a rapid and transient accumulation of ROS in Arabidopsis peaking after 1 h followed by a second oxidative burst after about 6 h (Xie et al., 2011). The interpretation was that HY1 plays an important role in salt stress-signaling and that this pathway requires the participation of AtRBOHD-derived ROS from peak II. More recently, AtRBOHF was implicated in protecting shoot cells from transpiration-dependent accumulation of excess Na^+ (Jiang et al., 2012). ROS generated by AtRBOHF have a specific role in regulating Na^+ accumulation and soil-salinity tolerance (Jiang et al., 2012). Sakamoto et al. (2008) found that the expression of RBOHC/D/F genes is induced in response to salt stress in wild-type plants. The salt-responsive induction of *RBOH* accumulation was markedly suppressed in the *itn1-1* mutant. This mutant revealed

increased tolerance to NaCl by affecting the ABA-mediated ROS production (Sakamoto et al., 2008). This suggest that the *itn1-1*, an ankyrin-repeat containing membrane protein, suppresses induction of the NADPH oxidase genes in response to salt stress, resulting in lower levels of H_2O_2, and that this may cause the salt-tolerant phenotype. The comparison of the salinity response of Arabidopsis wild type and *AtrbohD/F* double mutant indicates that the early H_2O_2 generation by NADPH oxidase triggers the antioxidant response in *A. thaliana*. The fortified antioxidant defense counteracts the subsequent ROS production and thereby mitigates the salt stress-derived injuries (Rejeb et al., 2014). According to Leshem et al. (2007), the salt-induced ROS production by NADPH oxidase in endosomes was suppressed in the phosphatidylinositol 3 kinase mutant (*pi3k*) and this mechanism causes a reduction in oxidative stress. In this experiment RBOH produces $O_2^{\bullet-}$ which is coordinated by the phospholipid-regulated signaling pathway and takes part in signal transduction in response to salt stress (Leshem et al., 2007). RBOHs also contribute to proline accumulation in response to salt or mannitol stress (Rejeb et al., 2015b). The timing and the magnitude of RBOH-dependent $O_2^{\bullet-}$-generation participate in inducing the salinity acclimation response on the one hand, but also in accelerating the deleterious effect of excessive ROS accumulation leading to salinity toxicity on the other hand.

RBOH-dependent ROS triggers signal transduction pathways and mediates local and systemic signaling (Miller et al., 2009; Marino et al., 2012). The initial Ca^{2+}-influx through plasma membrane ion channels and the RBOH-mediated production of ROS are synergistically activated by the binding of Ca^{2+} to EF-hand motifs as well as Ca^{2+}-dependent phosphorylation. Both mechanisms participate in regulating acclimation to salinity in plants including halophytes (Kurusu et al., 2015). RBOHs are central players in the Ca^{2+}-ROS signaling network triggered by their phosphorylation during stress adaptation (Kimura et al., 2012; Gilroy et al., 2014). This Ca^{2+}- and ROS-dependent signaling network regulates downstream events such as the Ca^{2+}-dependent activation of the Na^+/H^+-exchangers SOS1 and NHX1, Na^+ efflux from the cytosol, xylem loading of Na^+, Na^+ exclusion from leaves, induction of osmolyte synthesis and osmo-protective proteins, and overall the maintenance of cytosolic ion balance (Reguera et al., 2014). Work with *atrbohD/F* double mutants revealed that ROS are rate-limiting second messengers in ABA signaling. AtRBOHD/F function in ABA signal transduction in guard cell (Kwak et al., 2003). Following ABA perception in guard cells, active SnRK2 kinases such as OST1 (OPEN STOMATA 1) phosphorylate RBOHF and thereby stimulate ROS accumulation which in turn activates two MAPKs and regulates ABA-mediated stomatal closure (Danquah et al., 2014). RBOH-dependent ROS have been detected in vesicles in response to salt stress or during abscisic acid (ABA)-induced stomatal closure (Leshem et al., 2007). The different signaling mechanisms in RBOH activity control and the direct and indirect involvement in multiple downstream, processes characterize RBOH as a signaling hub for salinity acclimation.

NADP-DEPENDENT MALATE DEHYDROGENASE (NADP-MDH) AND THE MALATE VALVE

$O_2^{\bullet-}$ is generated in the PET by transfer of electrons from ferredoxin or reduced plastoquinone to O_2, particularly if $NADP^+$ or other terminal electron acceptors are unavailable. Likewise strong NADH feeding into the respiratory chain eases $O_2^{\bullet-}$ generation as long as AOS is not activated. Thus, reoxidation of NADPH to $NADP^+$ is important for balancing the ATP/NAD(P)H ratio and maintaining redox homeostasis. The photosynthesizing chloroplast employs diverse mechanisms to balance the rates of ATP and NADPH generation, e.g., by activating cyclic electron flow (CEF) which is under control of redox stimuli (Strand et al., 2015). Naturally such mechanisms do not allow for drainage of excess electrons if metabolic consumption is inhibited. Under such conditions activation of the malate-oxaloacetate (OAA) shuttle allows for transfer of reducing equivalents between cell compartments, e.g., under stress condition of plant (Heber, 1974; Taniguchi and Miyake, 2012; **Figure 3**). Another $NADP^+$-generating mechanism is chloroplastic GR which reduces GSSG released in the water-water cycle. Excess electrons from photosynthetic electron transport are used by TRX-regulated NADP-dependent malate dehydrogenase (MDH) to reduce OAA to malate, thus regenerating the electron acceptor $NADP^+$ (Scheibe et al., 2005). The resulting malate is subsequently translocated to the cytosol via the malate-OAA shuttle, where the interconversion of malate to OAA with concomitant reduction of NAD^+ to NADH is catalyzed by the cytosolic NAD-MDH (Hara et al., 2006). The NADH is fed into the RET. This allows for maintenance of chloroplast redox homeostasis and plays an important role in the short-term adjustment of the NADP(H) redox state also in response to salinity stress (Scheibe et al., 2005). In line with this scenario, Cushman (1993) measured a more than twofold increase of chloroplast NADP-MDH transcript level in leaves of *Mesembryanthemum crystallinum* under salt stress. In a recent study, Gawronska et al. (2013) described a set of protective strategies which accompany acclimation to salinity in the halophytic species *M. crystallinum*. The malate valve appears to be of prime importance. Salinity stress increases activities of NAD-MDH in whole tissue extract, and specifically mitochondrial NAD-MDH and chloroplast NADP-MDH in salt tolerant rice cv CSR-1 and CSR-3 whereas the activities were inhibited in salt sensitive cultivars (Kumar et al., 2000). Apparently the malate valve-dependent redox balance constitutes an important mechanism in salt acclimation.

PLASTID TERMINAL OXIDASE (PTOX)

The plastid terminal oxidase (PTOX) is a nucleus-encoded plastid-located plastoquinone (PQ)-O_2 oxidoreductase (plastoquinol oxidase) which transfers electrons from PQ to O_2 and forms H_2O (Carol et al., 1999). It represents the key component of an alternative electron pathway which involves

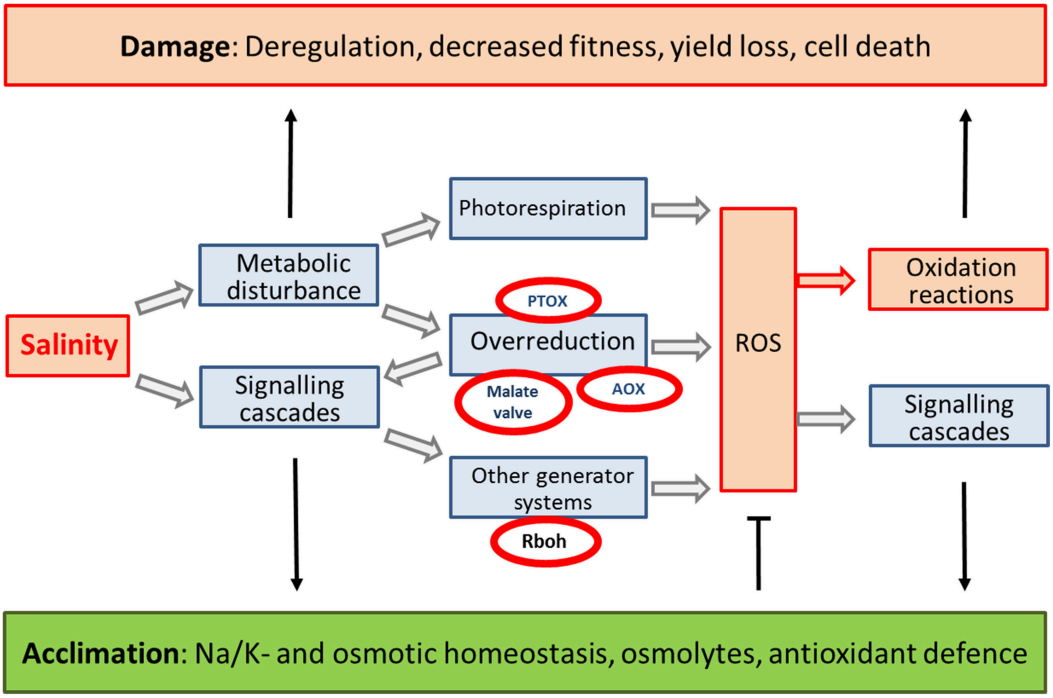

FIGURE 4 | Circuitry of redox and ROS-related events in salinity stress response. Salinity stress causes metabolic imbalances and activates signaling pathways. The metabolic imbalances increase the ROS generation e.g., by enhanced photorespiration or cause overreduction of PET and RET as described in the review. The latter is modulated by AOX, PTOX and malate valve. Signaling activates RBOH. Accumulating ROS either cause excessive oxidation reactions leading to damage or via redox- and ROS-dependent signaling and regulation allow for proper acclimation.

the reduction of PQ by NAD(P)H dehydrogenase (NDH) and the oxidation of reduced PQ by PTOX (Peltier and Cournac, 2002). PTOX is involved in chloroplast development and is suggested to act as safety valve to prevent the over-reduction of the photosynthetic machinery under stress conditions (Carol et al., 1999). However, additional features of PTOX have questioned the safety valve function owing to its ability to produce ROS under stress (Heyno et al., 2009; Feilke et al., 2014; Yu et al., 2014). When the PQ pool is highly reduced, PTOX itself produces superoxide ($O_2^{\bullet-}$) in a side reaction, triggering retrograde signaling to the cytosol and altering expression of response genes needed for acclimation to the environment (Yu et al., 2014; Krieger-Liszkay and Feilke, 2016). Overexpression of PTOX in *A. thaliana* did not protect against light-induced photodamage (Rosso et al., 2006) which appears contradictory to a protective function of PTOX. Under excess light PTOX overexpressors generate $O_2^{\bullet-}$. If this $O_2^{\bullet-}$ is efficiently detoxified by the antioxidant system, then even this mechanism may act as a safety valve (Heyno et al., 2009). If the antioxidant system is overwhelmed then PTOX-generated $O_2^{\bullet-}$ would enhance damage formation. Overall, the function of PTOX appears to be Janus-like. PTOX-dependent ROS may damage the photosynthetic apparatus or contribute to its protection and regulation.

Up to 10% of the photochemically produced O_2 in *Haematococcus pluvialis* was consumed by PTOX via the astaxanthin biosynthesis pathway. This pathway could lower the oxygen partial pressure and thereby reduce ROS release in the alga cell (Li et al., 2008). PTOX protects PSII by moving the site of ROS production from the appressed membranes with the majority of PSII to the nonappressed membranes where PTOX is located (Joët et al., 2002). In stressed plants, PTOX plays a regulatory role in carotenoid biosynthesis and in PQ oxidase activity in chlororespiration to re-oxidize reduced PQ (Bennoun, 1982; Kuntz, 2004; Campos et al., 2015). PTOX also controls the stromal redox poise (Trouillard et al., 2012). The abundance of PTOX is positively correlated with the intensity of salinity stress (Ivanov et al., 2012; Nawrocki et al., 2015). PTOX levels increase in salt stressed plants. PTOX-dependent electron drainage accounted for up to 30% of total PSII electron flow which provides strong evidence for its role as safety valve relative to photorespiration (Stepien and Johnson, 2009). Intensive H_2O_2 generation in photorespiration stimulated the activity of PTOX. This regulation seems to anticipate and subsequently counteract the effects of aggravating salinity stress. In a converse manner, inhibition of PTOX stimulated the H_2O_2 formation which might be important as signaling cue to initiate acclimation of halophytic *Thellungiella* plants (Wiciarza et al., 2015). PTOX attaches to the thylakoids at alkaline pH. This led to the suggestion that PTOX senses excessive alkalization of the stroma, subsequently attaches to the thylakoids and facilitates reoxidation of the PQ pool. By this mechanism overreduction of the photosynthetic electron

transport is avoided and ROS generation decreased (Feilke et al., 2016).

OTHER MECHANISMS OF ELECTRON DRAINAGE UNDER SALINITY

The accumulation of organic osmolytes, such as proline, glycine betaine, sugar alcohols, polyamines, and proteins e.g., from the late embryogenesis abundant (LEA) superfamily, in plasmatic compartments balances the osmotic potentials and maintains the low intracellular water potential of plants. Due to their compatible nature, these osmolytes counteract the harmful effects of ionic and osmotic stress (Verslues et al., 2006). Proline plays a crucial role in osmotic adjustment and acts as ROS scavenger, redox buffer, molecular chaperone which stabilizes proteins and membrane structures under stress (Matysik et al., 2002; Ashraf and Foolad, 2007). Proline synthesis via the glutamate pathway consumes 2 mol NADPH per mol proline and thus drains electrons from the chloroplast and buffers the cell reduction state (Hare and Cress, 1997). Accumulation of proline in leaves upon salt stress allows for continued carbon reduction and counteracts photoinhibition and excess ROS production. In the mitochondria proline is catabolized and the reducing power can be dissipated by RET coupled to AOX bypassing complex III and IV. Analyses of transcriptional regulation and knockout mutants indicate that the Arabidopsis Δ1-pyrroline-5-carboxylate synthetase1 (*P5CS1*) genes, the controlling step of proline synthesis, have clearly distinct functions. *P5CS1* is strongly induced under high salinity (Szekely et al., 2008). Consistent with the upregulation of *AtP5CS1*, *p5cs1* knockout mutants have greatly reduced proline levels during salt stress, resulting in reduced growth and altered ROS levels, suggesting that they are hypersensitive to salt (Szekely et al., 2008). Similar to proline, glycine betaine is an organic osmolyte synthesized by several plant families to balance the osmotic potential of intracellular compartments (Chen and Murata, 2011) under salinity. The plant pathway of synthesis consumes two electrons as net balance (Sakamoto and Murata, 2000). Activation of antioxidant mechanisms by proline and glycine betaine during salinity has been studied using tobacco bright yellow-2 suspension cultured cells (Hoque et al., 2007; Banu et al., 2009). Salinity significantly decreased the levels of reduced ascorbic acid and GSH, and the activity of water-water-cycle enzymes, and exogenous application of proline or glycine betaine increased the activity of these enzymes (Hoque et al., 2007). These results suggest a role of proline and glycine betaine in the regulation of antioxidant enzymes during salinity.

The last example of electron drainage with major significance for salinity is the pathway of photorespiration. Salinity-induced stomatal closure in moderately salt-stressed leaves leads to a drop in intercellular CO_2 concentration, increased oxygenation reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase, enhanced formation of photorespiratory metabolites such as phosphoglycolate, glycine and serine, and concomitant increase in H_2O_2 release in the peroxisomes, and CO_2 and NADH in the mitochondrion (Di Martino et al., 1999). The maintenance of electron transport by photorespiration in CO_2-free air corroborates the significance of photorespiration in salt-stressed leaves (Di Martino et al., 1999). Sustained rates of electron transport due to photorespiration and the formation of zeaxanthin during salt stress probably mitigate photoinhibitory damage (Sharma and Hall, 1992). The xanthophyll cycle is known as dynamic photoinhibition process that prevents overexcitation of the photosynthetic apparatus by dissipation of excess excitation energy (Krinsky, 1989; Niyogi et al., 1998). But, photorespiration also releases H_2O_2 in the peroxisomes as outlined above. The antioxidant system in the peroxisome efficiently detoxifies the photorespiratory H_2O_2. Jiménez et al. (1997) reported the presence of APX and MDHAR in peroxisomal membranes and argued that the membrane-bound antioxidant enzymes protect against H_2O_2 leaking out of peroxisomes. The photorespiratory NADH can be used in complex IV dependent RET for ATP synthesis or dissipated by AOX. This section on alternative drainage mechanisms only provides examples of involved pathways and cannot provide a comprehensive view.

CONCLUSIONS AND OUTLOOK

Maintenance of redox homeostasis is central to plant survival under salinity stress. Successful acclimation to saline growth conditions involves control of generation systems and tuning of antioxidant mechanisms. Under normal growth conditions generation and scavenging of ROS, and repair of damage are balanced. Salinity interferes with metabolism by ionic and osmotic effects and alters the redox and ROS state of the cell (**Figure 4**). This review demonstrates the flexible adjustment of each of the steps in response to salinity. It is striking that the described redox and ROS-related mechanisms of defense under salinity fit to the defense repertoire under other stresses such as photooxidative conditions. The specificity comes from the qualitative and quantitative use of stress-specific isoforms as discussed above. The principle differences between salt-sensitive and -tolerant genotypes includes specific symptomatic differences in redox and ROS generation. The central mechanisms realizing salt acclimation within the given tolerance range is selective short and long distance ion transport, safe ion compartmentation, synthesis of compatible solutes, and adjustment of osmotic homeostasis. As long as these mechanisms realize effective ion detoxification, deregulation of redox and ROS homeostasis is a minor component in stress acclimation. This changes if the salinity stress approaches the tolerance limits or during transition periods. Then the ultimate reason for decreased growth, reduced fitness and finally cell death are alteration in metabolism and excessive ROS accumulation (**Figure 4**). Some environmental conditions enhance salinity stress like periodical flooding with sea water, drought in saline environment or irrigation with saline water. The various mechanisms of dissipation of excess reducing power are mutually dependent. *A. thaliana* lacking chloroplast malate dehydrogenase are phenotypically inconspicuous (Hebbelmann et al., 2012). Proline synthesis, increased photorespiration and activation of

thiol peroxidase appear to compensate for the deficiency in NADP-MDH in high light treated *nadph-mdh* plants. This example shows the flexibility and partial redundancy of processes to control ROS release due to excessively accumulating reducing power and control of antioxidant defense which also contributes to salt stress acclimation. As long as the homeostasis mechanisms are functional and control the metabolic imbalances and keep ROS and redox deviations under control, small changes in redox- and ROS signatures are used to control gene expression, protein synthesis, metabolic activities and enable acclimation.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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A Different Pattern of Production and Scavenging of Reactive Oxygen Species in Halophytic *Eutrema salsugineum (Thellungiella salsuginea)* Plants in Comparison to *Arabidopsis thaliana* and Its Relation to Salt Stress Signaling

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Isolated thylakoids from halophytic *Eutrema salsugineum* (*Thellungiella salsuginea*) produces more H_2O_2 in comparison to glycophytic *Arabidopsis thaliana*. The first objective of this study was to verify whether this feature is relevant also to the intact chloroplasts and leaves. Enhanced H_2O_2 levels in chloroplasts and leaves of *E. salsugineum* were positively verified with several methods (electron microscopy, staining with Amplex Red and with diaminobenzidine). This effect was associated with a decreased ratio of $O_2^{\bullet -}/H_2O_2$ in *E. salsugineum* in comparison to *A. thaliana* as detected by electron paramagnetic resonance method. As a next step, we tested how this specific ROS signature of halophytic species affects the antioxidant status and down-stream components of ROS signaling. Comparison of enzymatic antioxidants revealed a decreased activity of ascorbate peroxidase (APX), enhanced activity of glutathione peroxidase, and the presence of thylakoid-bound forms of iron superoxide dismutase (FeSOD) and APX in *E. salsugineum*. These cues were, however, independent from application of salt stress. The typical H_2O_2-dependent cellular responses, namely the levels of glucosinolates and stress-related hormones were determined. The total glucosinolate content in *E. salsugineum* water-treated leaves was higher than in *A. thaliana* and increased after salinity treatment. Treatment with salinity up-regulated all of tested stress hormones, their precursors and catabolites [abscisic acid (ABA), dihydrophaseic acid, phaseic acid, 1-aminocyclopropane-1-carboxylic acid, salicylic acid, jasmonic acid, *cis*-(+)-12-oxo-phytodienoic acid and jasmonoyl-L-isoleucine] in *A. thaliana*, whereas in *E. salsugineum* only a stimulation in ethylene synthesis and ABA catabolism was noted. Obtained results suggest that constitutively enhanced H_2O_2

generation in chloroplasts of *E. salsugineum* might be a crucial component of stress-prepardeness of this halophytic species. It shapes a very efficient antioxidant protection (in which glucosinolates might play a specific role) and a fine tuning of hormonal signaling to suppress the cell death program directed by jasmonate pathway.

Keywords: chloroplast, glucosinolates, halophyte, hydrogen peroxide, salinity, stress hormones

INTRODUCTION

Reactive oxygen species (ROS) are intriguing molecules, which are toxic to the biological structures but also play a signaling role in controlling plant growth, development and stress responses (for a recent review, see Suzuki et al., 2012; Baxter et al., 2014). They accompany the basal metabolic fluxes of aerobic organisms during the whole ontogeny. Considering the precise and multilevel control of metabolic changes it might not be surprising that ROS coming from the various cellular and extracellular sites seem to have their specific signaling targets (op den Camp et al., 2003; Avsian-Kretchmer et al., 2004; Gadjev et al., 2006; Geisler et al., 2006; Laloi et al., 2007). In plants several ROS, such as H_2O_2, $O_2^{\bullet -}$ and $^1O_2$, are formed in chloroplasts aside the photosynthetic electron transport. They, in turn, affect the nuclear gene expression to adjust the photosynthesis to changing environment. The most stable form, which is assumed to leave this organelle and evoke the effects on the nuclear genes, is H_2O_2 (Mubarakshina et al., 2010; Borisova et al., 2012).

In an attempt to recognize the signaling effects of H_2O_2 originating from chloroplasts, *Arabidopsis* mutant overexpressing glycolate oxidase in chloroplasts (GO5 plants) has been developed (Fahnenstich et al., 2008). In the photorespiratory conditions GO5 mutants produce H_2O_2 in chloroplasts instead of in peroxisomes. Transcript profiling of GO5 plants (Balazadeh et al., 2012) identified the H_2O_2 regulated genes and transcription factors, whereas further work of Sewelam et al. (2014) underpinned the top 20 genes specifically up-regulated by H_2O_2 produced in chloroplasts. These studies proved that H_2O_2 can trigger different responses depending on the subcellular site of its production.

Recently, we demonstrated that thylakoids isolated from a highly stress resistant species *Eutrema salsugineum (Thellungiella salsuginea)* are capable of the enhanced production of H_2O_2, in comparison to *Arabidopsis thaliana*, already in the absence of stress (Wiciarz et al., 2015). This creates the opportunity to unravel the signaling action of chloroplast H_2O_2 generation in the natural system. *E. salsugineum* tolerates extreme salinity, cold, drought, ozone and over the last years this species became a plant model of stress resistance well-comparable with the *Arabidopsis* genome (Inan et al., 2004; Li et al., 2006; Amtmann, 2009; Hou and Bartels, 2015). So far, several studies focused on the discovery of transcriptomic footprints of high stress resistance in *E. salsugineum*. These studies showed that several stress-associated genes in *E. salsugineum* have a constitutively higher expression in comparison with *A. thaliana* already in the absence of stress (Inan et al., 2004; Taji et al., 2004; Gong et al., 2005). The group of up-regulated genes includes, for example, those involved in abscisic acid (ABA) biosynthesis and signaling (Taji et al., 2004; Gong et al., 2005). In contrast, after stress treatment, only a slight change in gene expression was detected in *E. salsugineum* in comparison with a great activation of transcription in *A. thaliana* plants (Taji et al., 2004; Li et al., 2006; Wong et al., 2006). Also, a comparative proteomics of *A. thaliana* and *E. salsugineum* salt responses revealed more changes in protein abundance in *Arabidopsis* than in *Eutrema* (Pang et al., 2010). Combined, these results indicate so called 'stress preparedness' of *E. salsugineum*, which supports its halophytic nature. Comparison of metabolite profiles of these two species following salt stress revealed a significant differences (Arbona et al., 2010). This suggests, that an adjustment of metabolism and activation of the already present enzymatic machinery serves as a faster and more efficient strategy to cope with stress than synthesis of new proteins.

A goal of this study was to verify whether enhanced generation of H_2O_2 in thylakoids of halophytic *Eutrema* in comparison to glycophytic *A. thaliana* is significant also *in vivo*. As a next objective, it was tested how this situation influences oxidative damage, antioxidant system and hormonal signaling in the control conditions and after a salinity stress.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (Col-0) and *E. salsugineum* (*T. salsuginea,* salt cress) ecotype Shandong were grown from seeds in the soil culture under irrigation with tap water. Seeds were obtained from the Nottingham *Arabidopsis* Stock Centre, UK. Plants were cultivated in the phytotron chamber at temperatures of 18°/16°C day/night, photoperiod 10/14 h, irradiance of ab. 220 μmol m^{-2} s^{-1} and RH ∼50%. Both species were adapted to these conditions for at least three generations. Because of delay in growth of *E. salsugineum* in comparison to *A. thaliana*, as reported earlier (Inan et al., 2004; Stepien and Johnson, 2009), to compare rosettes at the same developmental stage, 4-weeks-old *A. thaliana* and 5-weeks-old *E. salsugineum* plants were taken for experiments. To evoke a mild salinity-stress plants were irrigated with 0.15 and 0.3 M NaCl solutions for *A. thaliana* and *E. salsugineum*, respectively, while watered plants served as controls. After 7 days of NaCl treatment complete rosettes were collected, frozen in liquid nitrogen and stored at −80°C until further use (unless stated otherwise).

Thylakoid Membrane Preparation

Thylakoids were isolated as described earlier (Wiciarz et al., 2015). Shortly, leaves were homogenized in medium containing 50 mM HEPES-KOH (pH 7.6), 330 mM sorbitol (control plants)

and 495 mM sorbitol (NaCl-treated plants) respectively, 1 mM $MgCl_2$, 2 mM Na_2EDTA, 5 mM sodium ascorbate and 0.01% (w/v) fatty acid-free bovine serum albumine. After centrifugation for 4 min at 4000 × *g* the pellet was resuspended in 50 mM HEPES-KOH (pH 7.6), 5 mM sorbitol, 5 mM $MgCl_2$ and centrifuged again. Then the pellet was washed and resuspended in 50 mM HEPES-KOH (pH 7.6), 330 mM sorbitol, 10 mM $MgCl_2$, 20 mM NaCl, 2.5 mM Na_2EDTA, 10 mM $NaHCO_3$. The chlorophyll concentration was estimated spectrophotometrically according to Lichtenthaler and Buschmann (2001).

Electron Paramagnetic Resonance (EPR) Measurements

Production of $O_2^{\bullet -}$ and H_2O_2 by thylakoids from *A. thaliana* and *E. salsugineum* water-treated plants was detected by electron paramagnetic resonance (EPR) spin-trapping spectroscopy using DMPO (5,5-dimethyl-pyrroline *N*-oxide; Sigma-Aldrich, USA) and POBN [α-(4-pyridyl-1-oxide)-*N*-tertbutylnitrone; Sigma-Aldrich] as the spin trap, respectively, as described earlier (Jajić et al., 2015). Shortly, for $O_2^{\bullet -}$ detection, isolated thylakoids (chlorophyll concentration 200 μg mL^{-1}) were mixed with DMPO to a final concentration 50 mM, transferred to a flat cell and illuminated for 5 min at 500 μmol quanta m^{-2} s^{-1} within the EPR spectrometer MiniScope MS300 (Magnettech GmbH, Germany). For H_2O_2 detection, the H_2O_2-derived hydroxyl radical after the initiation of the Fenton reaction was measured. Isolated thylakoids (concentration of chlorophyll 150 μg mL^{-1}) in a reaction medium pH 7.6 containing 0.4 M sucrose, 20 mM NaCl, 5 mM $MgCl_2$, 10 mM Hepes-KOH were illuminated with white light source LS2 (Hansatech, UK) for 3 min at 500 μmol quanta m^{-2} s^{-1}. Next, 50 mM POBN, 50 μM FeEDTA and 4% ethanol were added and incubated for 3 min, transferred to glass capillaries and measured using an EPR spectrometer. To check the influence of enzymatic scavengers on H_2O_2 production, measurements were performed in presence and absence of 5 mM sodium azide (inhibitor of heme containing enzymes).

H_2O_2 Detection

Ultrastructural localization of H_2O_2 was visualized in transmission electron microscopy (TEM) via electron-dense precipitates of cerium perhydroxides [$Ce(OH)_2OOH$ and $Ce(OH)_3OOH$] formed after the reaction of cerium chloride ($CeCl_3$) with endogenous H_2O_2 (Bestwick et al., 1997). After 1 h of light leaves of *A. thaliana* and *E. salsugineum* from water-treated plants were cut into small pieces (∼5 mm). Leaf fragments were immediately infiltrated and then incubated for 1 h with a 0.5 M morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0), containing 5 mM $CeCl_3$ (Libik-Konieczny et al., 2015). The controls were tissue samples incubated in MOPS buffer. Then, tissues were quickly washed in the 0.5 M MOPS and fixed in 2.5% (w/v) formaldehyde (prepared from paraformaldehyde) and a 2.5% (v/v) glutaraldehyde in 50 mM cacodylate buffer (pH 7.0) for 4 h at room temperature. The procedure for preparing the samples for TEM was as described earlier (Kozieradzka-Kiszkurno and Płachno, 2012). The material was dehydrated in a series of graded acetone and embedded in Spurr Low-Viscosity Embedding Kit (Polysciences, Germany). Ultrathin (60–90 nm) sections were cut with a diamond knife on a Leica EM UC7 ultramicrotome. The sections were stained with uranyl acetate and lead citrate and then viewed using a Philips CM 100 TEM at 75 kV.

Histochemical localization of H_2O_2 production in leaves was determined using the DAB (3,3-diaminobenzidine) staining technique according to Libik-Konieczny et al. (2015). Leaves of *E. salsugineum* and *A. thaliana* water-treated plants were infiltrated with a solution of 1 mg mL^{-1} DAB (Sigma-Aldrich) prepared in water. Incubation was carried for 4 h in the dark at room temperature.

SDS-PAGE and Immunoblot Analysis

Leaf soluble proteins were extracted with 0.1 M phosphate buffer pH 7.5 containing 1 mM dithiothreitol, 2% (w/v) polyvinylpolypyrrolidone and protease inhibitor cocktail (Sigma-Aldrich). Homogenates were centrifuged 10 min at 10000 × *g*. Protein concentration in supernatant was estimated using Roti®-Nanoquant Protein quantitation assay (Carl Roth, Germany). SDS-PAGE as well as immunoblotting were performed as described earlier (Niewiadomska et al., 2009). Thylakoid membranes (TMs) and soluble proteins were dissolved in denaturating buffer and heated 20 min at 99°C (soluble proteins) or 5 min at 75°C (TMs). After electrophoresis, the separated proteins were blotted onto nitrocellulose membranes and probed with polyclonal antibodies raised against: ascorbate peroxidase (APX; anti-APX); glutathione peroxidase (GPX; anti-GPX); iron superoxide dismutase (FeSOD; anti-FeSOD); gamma glutamylcysteine synthase, γ -ECS (anti-γ -ECS); peroxiredoxins Q, PrxQ (anti-PrxQ). All antibodies were purchased from Agrisera (Sweden).

Determination of Lipid Peroxidation

Lipid peroxidation in leaves and isolated thylakoids of *A. thaliana* and *E. salsugineum* water-treated plants was assessed by measuring the malondialdehyde (MDA) content using high-performance liquid chromatography (HPLC) as described by Rastogi et al. (2014). Shortly, leaf samples were ground in a mortar with chilled 80% (v/v) ethanol. The extract was then centrifuged (10000 × *g* for 2 min) and the supernatant was further used. Samples were mixed with equal amount of reaction mixture containing 20% (w/v) trichloroacetic acid, 0.01% (w/v) butylated hydroxytoluene and 0.65% (v/v) thiobarbituric acid (TBA). After heating at 95°C for 20 min and centrifugation the MDA-$(TBA)_2$ adduct was separated and quantified by the HPLC. The elution buffer was 50 mM KH_2PO_4 (pH 7.0)/methanol (65:35, v/v). The retention time was 5 min and flow rate of 0.5 mL min^{-1} with detection at 530 nm. Tetraethoxy-propane (Sigma-Aldrich) was used as a standard.

Quantification of H_2O_2 Concentration

An Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies/Thermo Fisher Scientific, USA) was used to measure H_2O_2 content in *A. thaliana* and *E. salsugineum* leaves after water irrigation. Leaves (0.1 g) frozen in liquid

nitrogen were ground with 0.5 M sodium phosphate buffer, pH 7.4, and centrifuged 10 min at 10,000 × *g*. Proteins were removed from the extracts using Amicon Ultra Centrifugal Filters (Merck Millipore, USA) and the filtrate (25 μL) was incubated for 30 min in dark conditions with 50 mM Amplex Red reagent and 0.2 units mL^{-1} horseradish peroxidase. Excitation was measured at 530 nm and fluorescence detection at 590 nm with a microplate reader Synergy 2 (BioTek, USA). The hydrogen peroxide concentration was estimated by comparison with standard curve (0–10 μM) of H_2O_2. The experiments were repeated three times independently, each time in triplicate.

Determination of Antioxidant Enzyme Activities

Soluble leaf proteins were extracted with 0.1 M phosphate buffer pH 7.5 [catalase (CAT); SOD], 50 mM phosphate buffer pH 7.0 (GPX) or 0.1 M phosphate buffer pH 7.8 with 1 mM ascorbic acid (APX), in each case containing protease inhibitor cocktail (Sigma-Aldrich). After centrifugation 10 min at 10000 × *g* at 4°C soluble proteins were desalted using Amicon Ultra Centrifugal Filters (Merck Millipore). Protein concentration was estimated using Roti® -Nanoquant Protein quantitation assay (Carl Roth).

Catalase activity was measured according to the method described by Aebi (1984). The decomposition of 10 mM H_2O_2 in phosphate buffer (50 mM phosphate buffer pH 7.0) was monitored for 2 min at 240 nm. Calculations used an absorbance coefficient of 43 M^{-1} cm^{-1}.

Ascorbate peroxidase activity was determined according to Nakano and Asada (1981). The decrease in absorbance at 290 nm due to ascorbate oxidation was recorded. The assay mixture contained 20 μg proteins, 0.1 mM EDTA, 0.5 mM ascorbic acid, 50 mM and 0.1 mM H_2O_2 in phosphate buffer (pH 7.0). The absorbance was recorded after 3 min. The APX activity was calculated using the absorbance coefficient of 2.8 mM^{-1} cm^{-1}.

Glutathione peroxidase activity was determined as the decrease in absorbance at 340 nm due to the oxidation of NADPH ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) according to Hopkins and Tudhope (1973). The reaction mixture consisted of 50 mM phosphate buffer (pH 8.0) containing 0.5 mM EDTA, 0.125 mM NADPH, 1 mM reduced glutathione, 0.5 U/mL glutathione reductase (Sigma-Aldrich), 300 μM tert-butyl hydroperoxide and 20 μg proteins. The reaction was monitored for 3 min.

Glucosinolate Content

The total glucosinolates were extracted and assessed basically as described by Aghajanzadeh et al. (2014). Leaf samples (0.1 g) were boiled for 2 min in 3 ml 90% methanol. The extract was centrifuged (2500 × *g* for 2 min) and the residues were extracted again. The determination of total glucosinolate content was based on color complex formation between glucosinolates and sodium tetrachloropallade (II). The 60 μl of extract was incubated for 30 min in 1800 μl 2 mM Na_2PdCl_4 and absorbance of developed color was measured at 450 nm. The glucosinolate content was calculated using standard curve of sinigrin (0–3 mM; Sigma-Aldrich).

Plant Hormone Determination

For hormone analysis *A. thaliana* and *E. salsugineum* leaf samples were collected at midday. Extraction and analysis were performed according to Dobrev and Kamínek (2002) and Dobrev and Vankova (2012). Briefly, approximately 100 mg fresh samples were homogenized and extracted with methanol/water/formic acid (15/4/1, v/v/v). The following labeled internal standards (10 pmol per sample) were added: $^2H_6$-ABA, $^2H_3$-PA,$^2H_4$-SA, $^2H_5$-JA (Olchemim, Czech Republic). Extracts were purified using SPE-C18 column (SepPak-C18, Waters, Milford, MA, USA) and separated on a reverse phase-cation exchange column (Oasis-MCX, Waters). The hormone fraction was eluted with methanol, separated by HPLC (Ultimate 3000, Dionex/Thermo Fisher Scientific, Austria) and the hormones were quantified using a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems/MDS SCIEX, Foster City, CA, USA). The analyses were carried out in three biological replicates. The analysis of ABA and its catabolites DPA (dihydrophaseic acid) and PA (phaseic acid); ACC (1-aminocyclopropane-1-carboxylic acid), SA (salicylic acid), jasmonic acid (JA) and jasmonate precursor *cis*-OPDA [*cis*-(+)-12-oxo-phytodienoic acid] and jasmonoyl-L-isoleucine (JA-Ile) was performed.

Statistical Analysis

All analysis were calculated with Student's *t*-test. Significant differences are marked at $P \leq 0.05$.

RESULTS

Production of H_2O_2 in Isolated Thylakoids and in Leaves

In thylakoids from *A. thaliana* and *E. salsugineum* an opposite patterns of ROS production were demonstrated by EPR method (**Figure 1**). Illuminated thylakoids of *E. salsugineum* generated more H_2O_2, as detected indirectly with POBN spin trap which reacts with H_2O_2-derived hydroxyl radical (**Figures 1A,B**), but less $O_2^{\bullet -}$, as detected with DMPO spin trap (**Figures 1C,D**), in comparison to the thylakoids of *A. thaliana*. Addition of NaN_3 strongly increased the production of H_2O_2, but did not significantly influence generation of $O_2^{\bullet -}$ the superoxide anion radical.

To verify unequivocally, whether a difference in H_2O_2 generation between the two species is significant also for chloroplasts and leaves we used several methodological approaches. The presence of H_2O_2 in the chloroplasts has been tested with a specific $CeCl_3$ staining visualized by TEM. Chloroplasts of mesophyll cells from water-treated *A. thaliana* revealed no cerium perhydroxide deposits (**Figure 2A**), while salt stress associated H_2O_2 accumulation was visible as black precipitate spots (**Figure 2B**), mainly at the edges of granal thylakoids (**Figure 2C**). In contrast, in chloroplasts of *E.*

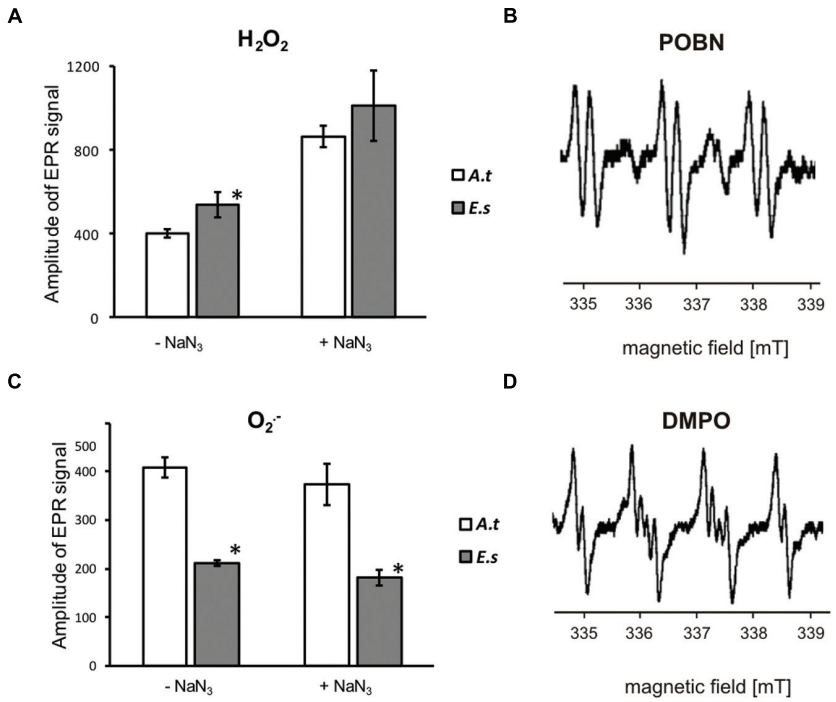

FIGURE 1 | Electron paramagnetic resonance (EPR) spin-trapping spectroscopy measurements of H_2O_2 (A,B) and $O_2^{\bullet-}$ (C,D) production by illuminated thylakoids isolated from water-treated *Arabidopsis thaliana* and *Eutrema salsugineum*. (A) The intensity of EPR signal of H_2O_2-derived hydroxyl radicals; **(B)** representative EPR spectra of the POBN adduct; **(C)** The intensity of superoxide anion radical measured with DMPO-OOH spin-trap; **(D)** representative EPR spectra of the DMPO adduct. The analyses were performed in the presence and absence of sodium azide (NaN_3). 'Asterisk' indicates significant difference from *A. thaliana*. Each data point represents the mean ± SD ($n = 3$). Significant differences between the averaged H_2O_2 production with and without NaN_3 in both species are not marked on the graph.

salsugineum an electron-dense precipitates were detectable already in control (**Figure 2D**), while more pronounced dark spots were visible after salt stress (**Figures 2E,F**). Another difference between *A. thaliana* and *E. salsugineum* detected by TEM was that a salinity-induced thylakoid swelling was detected only in *A. thaliana* chloroplasts (**Figure 2B**), while in *E. salsugineum* chloroplasts no signs of such destruction occurred (**Figures 2E,F**). Staining controls without $CeCl_3$ showed no electron-dense deposits in both species (data not shown).

Similarly, in leaves, enhanced content of H_2O_2 was documented in *E. salsugineum* than in *A. thaliana* (**Figure 3A**-staining with Amplex Red, and **Figure 3B**- staining with DAB). However, in spite of the high availability of this ROS a very low level of MDA was detected in *E. salsugineum* leaves, indicating a low extent of oxidative damage to membrane lipids (**Figure 4A**). This difference between the two species disappeared when isolated TMs were compared (**Figure 4B**). On this basis we formulated a hypothesis that enhanced leakage of H_2O_2 from plastids keeps the antioxidant system up-regulated, thereby preadapting plants to the salinity stress.

Comparison of Antioxidant System in *Arabidopsis* and *Eutrema*

The capacity of antioxidant system was compared in leaves of *A. thaliana* and *E. salsugineum* in the control conditions and after salinity stress. Due to the disturbed kinetics of enzymatic assays observed in crude leaf extracts (LEs) from *E. salsugineum*, the activities of H_2O_2 scavenging enzymes were analyzed in purified protein fractions. The activities of CAT were similar in both species in the control conditions and remained unchanged after salinity stress (**Figure 5A**). The activity of APX was lower in the *E. salsugineum* both in control and after salinity stress (**Figure 5B**). In contrast, the activity of thiol-dependent GPX was higher in *E. salsugineum* both in control and after a salinity stress (**Figure 5C**).

Considering the chloroplastic generation of H_2O_2 in *E. salsugineum*, the antioxidant system in this organelle might be of particular importance for this species. Therefore, we compared the antioxidant enzymes present at TMs and in the soluble fraction (LEs; **Figure 6**). The stromal form of APX (sAPX) was present in similar amount in both species, while distribution of thylakoid bound APX (tAPX) showed a clear difference between *A. thaliana* and *E. salsugineum*. This form was much more abundant in *Eutrema* both in controls and in salinity-treated plants, while it was barely detected in *A. thaliana*. Another differences between these two species was related to FeSOD. In *E. salsugineum,* FeSOD was less abundant in LEs, while it was much more abundant in TMs, when compared with *A. thaliana* (**Figure 6**). To get an insight into the level of thiols and thiol-dependent enzymes we compared the

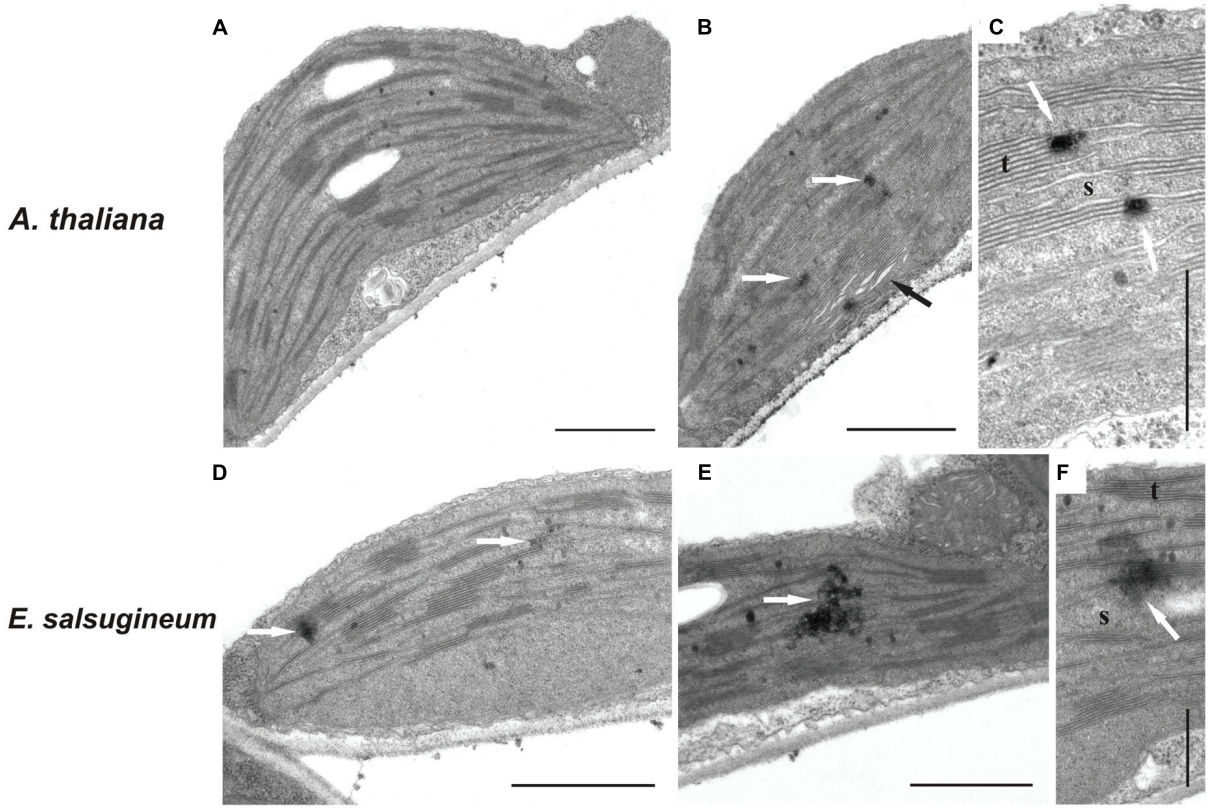

FIGURE 2 | Transmission electron microscopy (TEM) images of H_2O_2 accumulation in chloroplasts of *A. thaliana* and *E. salsugineum* plants treated with water and NaCl solution (0.15 and 0.3 M, respectively). The black spots (white arrows) represent H_2O_2 forming electron-dense cerium perhydroxide precipitates. *A. thaliana* chloroplasts from water-treated **(A)** and salt-stressed plants **(B,C)**. Black arrow in **(B)** points to the swelling of thylakoids. *E. salsugineum* chloroplasts from water-treated **(D)** and salt-stressed plants **(E,F)**. t, thylakoids; s, stroma. Bars = 1 μm **(A,B,D,E)**; Bars = 0.5 μm **(C,F)**.

amount of stromal peroxiredoxins Q (PrxQ) and the enzyme of reduced glutathione (GSH) synthesis, gamma glutamylcysteine synthase (γ-ECS). In *E. salsugineum* PrxQ were more abundant than in *A. thaliana*, and a slight decrease was noted after salinity treatment in both species, whilst γ-ECS was present at similar amount in both species (**Figure 6**). These pronounced differences between *E. salsugineum* and *A. thaliana* in the amounts of various ROS scavengers in chloroplasts suggest that there is a stronger need to protect photosynthetic membranes against ROS in the former species, already in the absence of stress.

Signaling Pathways Associated with ROS

It is well-documented that ROS signaling is interconnected with stress hormones (Baxter et al., 2014). Therefore, we analyzed the endogenous levels of stress-related hormones in *A. thaliana* and *E. salsugineum* plants grown in control conditions and changes evoked by salt-treatment. In control conditions, the concentrations of ABA and its catabolite PA were similar in leaves of both species (**Figures 7A,B**), while the level of another ABA catabolite, DPA was twofold lower in *E. salsugineum* (**Figure 7C**). Salt treatment caused an increase in ABA (twofold), DPA (twofold) and PA (threefold) in *A. thaliana*, whereas no significant changes (ABA, DPA), and a slight increase in (PA), were detected in *E. salsugineum* (**Figures 7A–C**). Concentrations of ethylene precursor ACC were similar in the two species in control conditions and underwent a similar increase due to salinity (**Figure 7D**). Concentrations of SA and jasmonates were much lower in the control *E. salsugineum* in comparison to *A. thaliana*: SA almost fourfold, JA by 4.5-fold, Ja-Ile by 2.4-fold and *cis*OPDA by 5.5-fold (**Figures 7E–H**). NaCl-treatment increased the levels of these hormones in *A. thaliana* (1.7-, 2.6-, 6.6-, 1.8-fold, respectively) but not in *E. salsugineum*, where their decreased concentrations were found (1.8-, 4-, 4-, 3.2-fold, respectively).

One of the highlights of H_2O_2 signaling from chloroplasts seems to be enhanced synthesis of glucosinolates, as demonstrated in GO5 plants (Balazadeh et al., 2012; Sewelam et al., 2014). To check this cue in our wild type model, we measured the total concentration of leaf glucosinolates. The total level of glucosinolates was enhanced in leaves of *E. salsugineum* in comparison with *A. thaliana* in control conditions, and a stronger increase was detected in *E. salsugineum* after salinity treatment (**Figure 8**).

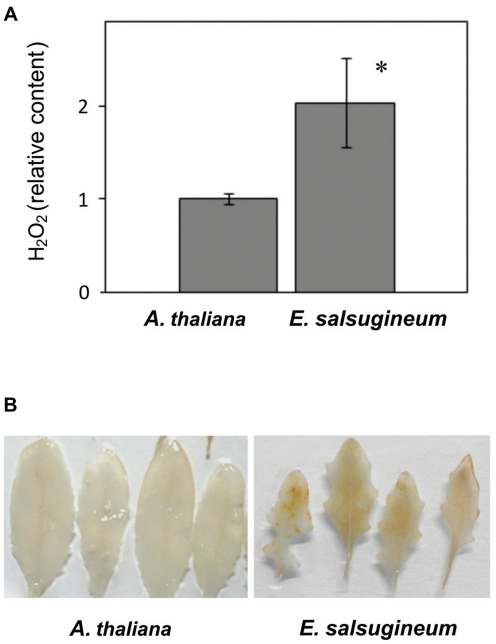

FIGURE 3 | Hydrogen peroxide (H_2O_2) accumulation in leaves from water-treated *A. thaliana* and *E. salsugineum* plants. (A) Content of H_2O_2 in deproteinated leaf extracts determined using the Amplex Red assay. Results were normalized to the average H_2O_2 content in *A. thaliana*. Data represent the mean ± SD ($n = 3$). 'Asterisk' indicates significant difference. **(B)** Visualization of H_2O_2 accumulation assessed by DAB staining of representative leaves.

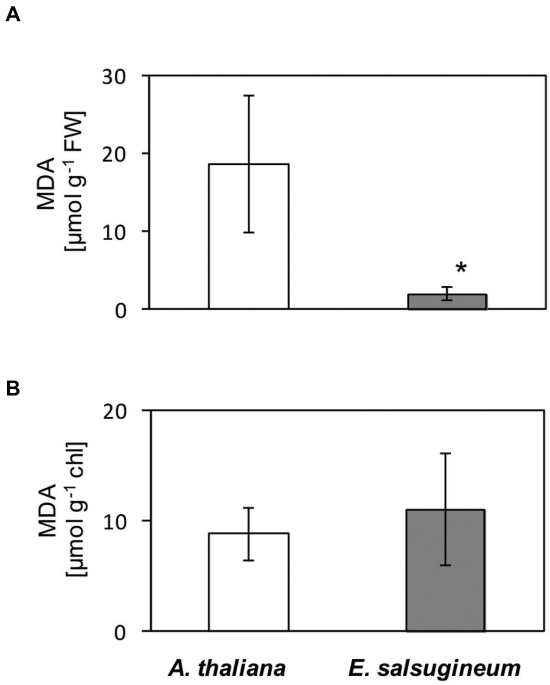

FIGURE 4 | Content of malondialdehyde (MDA) in leaves of water-treated *A. thaliana* and *E. salsugineum* plants (A) and isolated thylakoid membranes (TMs; B) assessed by high-performance liquid chromatography (HPLC). Each data point represents the mean ± SD ($n = 3$). 'Asterisk' indicates significant difference from *A. thaliana*.

DISCUSSION

In this work, we compared a halophytic *E. salsugineum* with glycophytic *A. thaliana* in regard to the chloroplastic H_2O_2 signature on the salt stress tolerance. As a first step, we verified whether a chloroplastic H_2O_2 signal, previously reported with use of isolated TMs (Wiciarz et al., 2015), persists in the relatively undamaged organelle and tissue.

Enhanced generation of H_2O_2 within the chloroplasts of control and stress-treated *Eutrema* was visualized by TEM, suggesting that a higher level of H_2O_2 persists in the whole chloroplasts. However, it is not uniformly distributed but spotted in the proximity of granal thylakoids, were it is produced. TEM images also revealed, that NaCl treatment caused thylakoid swelling only in *A. thaliana*. Thylakoid dilation is a well-documented symptom of salinity stress in plants (Niewiadomska et al., 2011; Yamane et al., 2012; Pottosin and Shabala, 2016), while structural integrity of *E. salsugineum* chloroplasts under mild NaCl-stress was previously shown by Chang et al. (2015). This lack of swelling might be attributed to the activity of thylakoid ion channels, and/or $K^+(Na^+)/H^+$ antiporters which control the ion homeostasis under salinity-stress (Pottosin and Shabala, 2016).

Although we did not find any direct evidence for an export of H_2O_2 from plastids, the total H_2O_2 level in leaf extracts was also elevated in *Eutrema* leaves. Hence, it is likely that a chloroplast H_2O_2 generation contributes considerably to this phenomenon. Our speculation is based on a well-accepted phenomenon that illuminated chloroplast is a major site of ROS production in the mesophyll cells (Asada, 2006). Moreover, a considerable leakage of H_2O_2 from healthy chloroplasts has been shown experimentally (Mubarakshina et al., 2010; Borisova et al., 2012).

Here, performed study revealed that an increased generation of H_2O_2 in halophytic species is associated by a decreased generation of $O_2^{\bullet -}$. Considering a much higher reactivity of $O_2^{\bullet -}$ than H_2O_2, this pattern of ROS production might shape a specific ROS signature important for so called stress-prepardeness of *Eutrema.* This speculation is supported by a much lower oxidative damage (MDA) in leaves of *E. salsugineum* than in *A. thaliana*. As argued by Tiwari et al. (2013), superoxide, but not H_2O_2, is required to evoke oxidative damage in photosynthetic membranes. This damage is mediated by the hydroxyl radical and is manifested by the appearance of carbon-centered radical. Experiments with the *flu A. thaliana* mutant and the *flu* overexpressing tAPX, enabled to demonstrate that H_2O_2 antagonizes the $^1O_2$-mediated cell death response and growth inhibition, which might be particularly advantageous under stress (Laloi et al., 2007). A rapid conversion of a more harmful ROS (such as $O_2^{\bullet -}$) into a longer-living and less toxic H_2O_2 has previously been concluded for halophytic plants on the basis of enhanced activity of superoxide dismutase (Bose et al., 2014). In halophytic *E. salsugineum* an operation

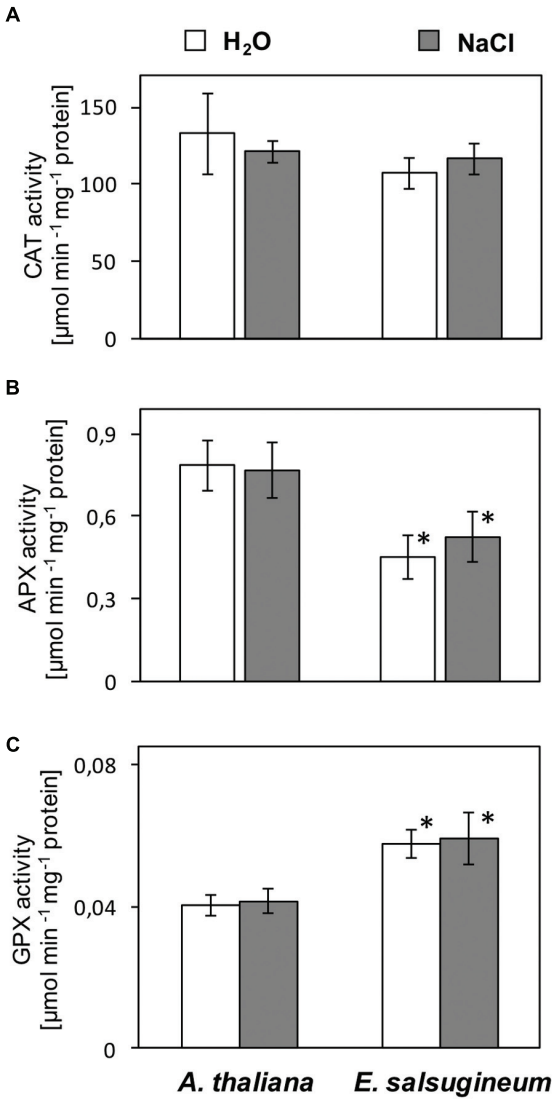

FIGURE 5 | Activities of CAT (A), APX (B), GPX (C) enzymes in the whole leaf cells of *A. thaliana* and *E. salsugineum* plants treated with water and NaCl solution (0.15 and 0.3 M, respectively). Data represent mean ± SD ($n = 3$). 'Asterisk' indicates significant difference from *A. thaliana* from the same treatment.

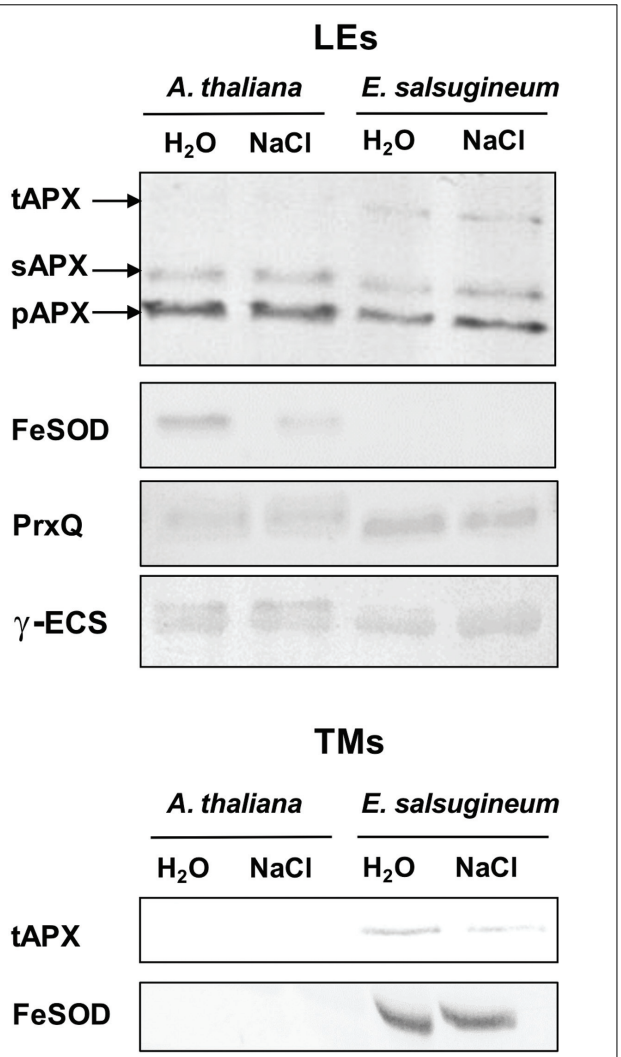

FIGURE 6 | Changes in the amount of APX (ascorbate peroxidase), FeSOD (iron superoxide dismutase), PrxQ (peroxiredoxin Q) and γ-ECS (gamma glutamylcysteine synthase) in *A. thaliana* and *E. salsugineum* plants treated with water and NaCl solution (0.15 and 0.3 M, respectively). Immunoblotting was performed with leaf extracts (LEs) and with thylakoid membranes (TMs). For visualization of APX, FeSOD and PrxQ in LE 40, 30, and 5 µg protein per lane was loaded, respectively. For visualization of APX, FeSOD and γ-ECS in TM 10 µg chlorophyll per lane was loaded. Blots are representative for three repetitions. sAPX, stromal APX; pAPX, peroxisomal APX; tAPX, thylakoid bound APX.

of the protective electron cycling around PSII (Stepien and Johnson, 2009; Wiciarz et al., 2015) seems to support that view. Such a cycle could be beneficial for plants in several ways. Firstly, it may create a more safe electron sink in comparison to the production of superoxide and its toxic derivatives. Secondly, an abundance of H_2O_2 may play a signaling role.

A growing body of data indicate that H_2O_2 triggers defense responses in plant cells (for a recent review, see Suzuki et al., 2012; Baxter et al., 2014; Bose et al., 2014; Ismail et al., 2014). In this respect, ROS formation in chloroplast may also act indirectly via strengthening or amplifying retrograde redox signals (Galvez-Valdivieso and Mullineaux, 2010). One of the targets of ROS signaling route under stress is an up-regulation of antioxidants (Fryer et al., 2003; Geisler et al., 2006). However, the activity of a major H_2O_2 scavenger CAT was similar in *E. salsugineum* and *A. thaliana* in controls and in salinity-treated plants, whereas APX activity was even decreased in *E. salsugineum*. This is in agreement with numerous reports on salt cress. The transcript profiling analysis with two *E. salsugineum* ecotypes did not detect any antioxidant genes among those specifically up-regulated by salinity (Taji et al., 2004; Wong et al., 2006). Also, a proteomic data did not detect any salinity-dependent stimulation of antioxidant enzymes in

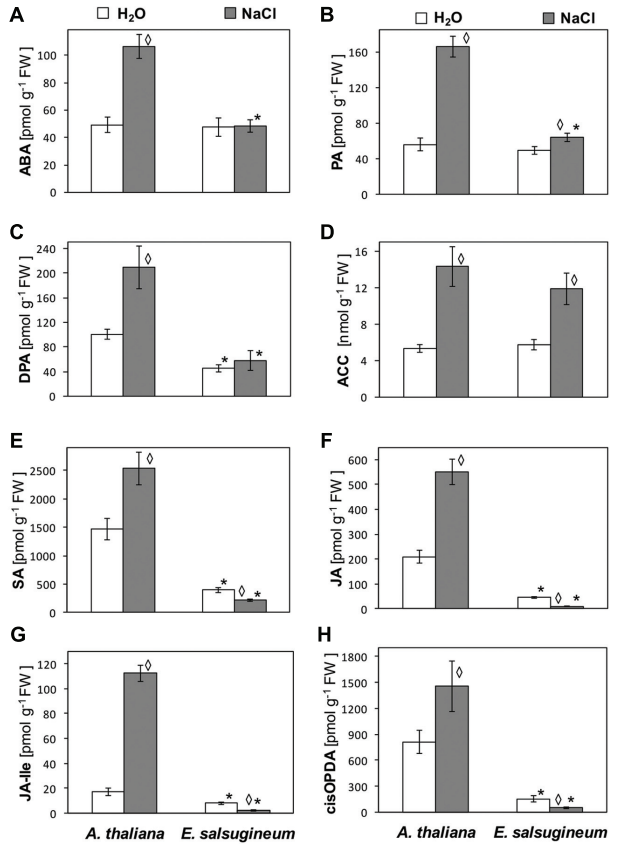

FIGURE 7 | Average content of stress-related hormones in leaves of *A. thaliana* and *E. salsugineum* plants treated with water and NaCl solution (0.15 and 0.3 M, respectively) assesed by LC–MS/MS. (A) ABA (abscisic acid). **(B)** DPA (dihydrophaseic acid). **(C)** PA (phaseic acid). **(D)** ACC (1-aminocyclopropane-1-carboxylic acid). **(E)** SA (salicylic acid). **(F)** JA (jasmonic acid). **(G)** JA-Ile (jasmonoyl-L-isoleucine). **(H)** *cis*OPDA (*cis*-12-oxo-phytodienioc acid). Each data point represents the mean ± SD ($n = 5$). 'Asterisk' indicates significant difference from *A. thaliana* from the same treatment. 'Diamond' indicates a significant difference between control and salinity-treated plants.

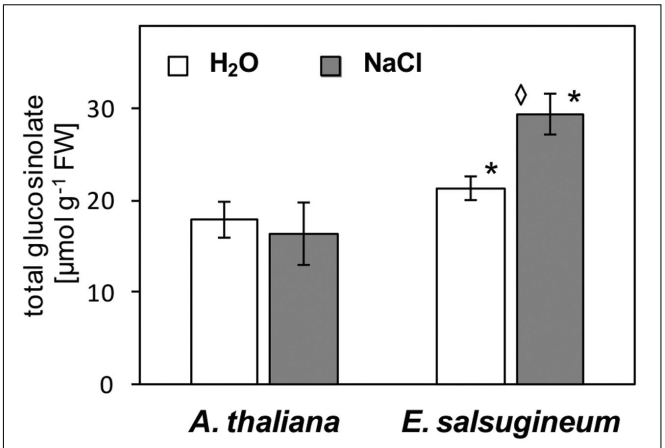

FIGURE 8 | Total glucosinolates content in leaves of *A. thaliana* and *E. salsugineum* plants treated with water and NaCl solution (0.15 and 0.3 M, respectively). Each data point represents the mean ± SD ($n = 3$). 'Asterisk' indicates significant difference from *A. thaliana* from the same treatment. 'Diamond' indicates significant difference between control and salinity-treated plants.

this species (Pang et al., 2010). At activity level M'rah et al. (2006) previously noted that stimulation of CAT activity in *E. salsugineum* occurred only under a mild salinity treatment, not greater than 100 mM, whereas some stimulation was related to total peroxidase activity (assessed with guaiacol as electron donor).

Intriguingly, all these data suggest that a high stress resistance of *Eutrema* does not rely on the major enzymatic H_2O_2 scavengers of mesophyll cells. We argue, that data obtained with a partially purified (desalted) protein extracts (as used here) give a more precise information about changes in the enzymatic activities in comparison to the data typically obtained with crude extracts. The latter are strongly influenced by a different availability of low molecular compounds, such as ascorbate in case of APX, and glutathione in case of GPX. Indeed, an increased pool of reduced ascorbate and of total glutathione was found due to salinity in *Eutrema*, but not in *Arabidopsis* (Wiciarz et al., 2016). This feature might be important in the context of H_2O_2 signaling. As suggested by Bose et al. (2014), decreased activities of a major H_2O_2 scavengers allow for a persistence of ROS signal in the cells. A fine-tuning of specific antioxidants seems to be more important for stress tolerance than a bulk changes in the antioxidant status (Jiang et al., 2007; Pang et al., 2010). We are, however, aware that a relation between ROS production and scavenging is highly dynamic while a situation reported here for *Eutrema* relates only to certain time point. But, on the other hand, it was verified with use of several methods and plants sets to be considered as physiologically relevant.

Among the enzymatic H_2O_2 scavengers only GPX was enhanced in *E. salsugineum*. This points to the importance of redox active thiols. GPXs reduce not only H_2O_2 but also organic hydroperoxides using reducing power of GSH. Hence, they may contribute to the dramatically reduced MDA level. A noteworthy stimulation of antioxidant defense associated with glutathione has previously been revealed in *E. salsugineum* by transcript profiling of Gong et al. (2005). This was represented by GPXs (*GPX2* and *GPX6*), glutaredoxins and glutathione-*S*-transferases (*GSTZ1* and *GSTU7*). Four out of eight GPXs found in *E. salsugineum* have been shown to respond to salt and osmotic stresses at both transcript and protein level (Gao et al., 2014). It is worthy to note here, that GPX may serve as a specific sensor and transducer of H_2O_2 signal. A dual function of GPX associated with both scavenging of ROS and transferring the H_2O_2 signal has been pointed out repeatedly (Delaunay et al., 2002; Miao et al., 2006). Moreover, a specific activation of *GPX1* promoter in tobacco by a chloroplast-generated H_2O_2 was documented by Avsian-Kretchmer et al. (2004). Whereas, study with *Arabidopsis gpx3* mutant demonstrated that GPX3 participates in the ABA and H_2O_2 signaling pathway regulating the stomatal aperture and resistance to drought (Miao et al., 2006).

Considering an enhanced generation of H_2O_2 and changed $O_2^{\bullet -}/H_2O_2$ ratio in chloroplasts, a major role might be attributed to the anti-oxidative protection within this organelle. Indeed, TMs of *Eutrema* appeared to be better protected against ROS in comparison with *Arabidopsis*, as indicated by a higher abundance of tAPX and FeSOD. This corresponds with enhanced level of *FeSOD* transcripts in the control *E. salsugineum*, reported by Taji et al. (2004). In regard to chloroplast stroma, we found an enhanced amount of PrxQ in *E. salsugineum*. These above-mentioned features of chloroplast antioxidant system are constitutive in *E. salsugineum*, which supports earlier conclusions on the stress preparedness of this species (Inan et al., 2004; Taji et al., 2004; Gong et al., 2005; Wong et al., 2006). They are also in line with the view that components of redox homeostasis in chloroplasts are crucial for salinity tolerance (Niewiadomska and Wiciarz, 2015; Uzilday et al., 2015). So far, recognized chloroplast redox players highly engaged in *E. salsugineum* are: adenosine 5′-phosphosulfate reductase involved in sulfate assimilation (Gong et al., 2005); thioredoxin CDSP32 (M'rah et al., 2007); 2-cys peroxiredoxin BAS1 (Gao et al., 2009) and plastid terminal oxidase (PTOX), an enzyme of chlororespiratory pathway (Stepien and Johnson, 2009; Wiciarz et al., 2015). In addition to these, also proteins associated with the Calvin-Benson cycle, such as a more active Rubisco (Wiciarz et al., 2015) and a more abundant glyceraldehyde 3-phosphate dehydrogenase (Pang et al., 2010; Chang et al., 2015) are likely to contribute to the improved redox balance in *E. salsugineum* by efficient recycling of ADP and $NADP^+$.

A very weak response to salinity stress at the level of tested antioxidants may raise a question: whether *E. salsugineum* (and *A. thaliana*) senses the salinity stress at all? This has been clarified by analysis of stress hormones. A massive increase in stress-related hormones (ABA, SA, JA, and ethylene precursor) was detected in salinity-treated *A. thaliana*. These hormones are responsible for several stress effects in plants, among them stomatal closure and retardation of growth, although, their action is quite complex and may lead either to survival or to cell death. In contrast, in *Eutrema* the only indicator of salinity-evoked stress was a stimulation of ethylene synthesis. Although, some enhancement in ABA catabolite PA, may suggest a previous ABA induction. Earlier study of Ellouzi et al. (2014) documented that levels of ABA, JA, and ACC were increased in *E. salsugineum* after 2 days of salt stress, while 400 mM NaCl treatment for more than 5 days caused minimal changes in ABA and JA concentrations (Taji et al., 2004; Arbona et al., 2010). In *E. salsugineum* the levels of lipid-based active jasmonates (JA and JA-Ile) were low and even decreased due to salinity-treatment. This effect seems to be closely related to salt-resistance of *Eutrema*. As shown by Ismail et al. (2014), an interplay between ABA and JA is of particular importance for plant's survival or death under salinity stress. A comparison of the two *Vitis* species differing in salt-stress tolerance revealed that in the more salt-tolerant *Vitis rupestris* ABA accumulated early and strongly suppressed the formation of JA-Ile, while in the salt-sensitive *V. vinifera* the accumulation of ABA was delayed and correlated which allow for accumulation of JA-Ile up to the high levels. A suppression of jasmonates in *E. salsugineum* might also be explained in accordance with the data obtained with AtGO5 mutant overproducing H_2O_2 in chloroplasts, in which a stimulated expression of several repressors of JA signaling pathway has occurred (Balazadeh et al., 2012). A very weak response to salinity at the level of ABA in *E. salsugineum* in contrast to *A. thaliana* is surprising in view of data on a higher expression of genes for ABA-biosynthesis and signaling (Taji et al., 2004; Gong et al., 2005; Wu et al., 2012) and an increased number of genes involved in ABA synthesis pathway in *Eutrema* genome (Wu et al., 2012). Possibly, in *E. salsugineum* the increased level of ABA is confined to the early stage of development or this hormone is very rapidly modulated in response to stress (indicated by slight increase in PA). A weak response of stress hormones to salinity in *E. salsugineum* supports the earlier conclusion of Hou and Bartels (2015), that comparing with *A. thaliana* the higher NaCl levels (600 mM) are required in *E. salsugineum* to trigger some defense reactions, such as increased transcription of two aldehyde dehydrogenase genes.

On the other hand, the importance of glucosinolates for stress-resistance of *E. salsugineum* is depicted by their enhanced level and a strong salinity-dependent increase. A great variation in the patterns of aliphatic, aromatic, and indole glucosinolate was documented in *E. salsugineum*, depending on the organ and developmental stage (Pang et al., 2012). However, a precise role of these compounds in stress tolerance is not clarified yet. An up-regulation of the synthesis of indole glucosinolates and phytoalexin camalexin was found as a specific feature of increased H_2O_2 signaling from chloroplasts in studies with *At*GO5 mutant made by Balazadeh et al. (2012) and Sewelam et al. (2014). However, *E. salsugineum* does not produce camalexin, but wasalexins A and B and methoxybrassenin B (Pedras and Adio, 2008). Intriguingly, as another support for a constitutive stress-preparedness of *Eutrema*, the three phytoalexins (indolyl-3-acetonitrile, caulilexin C, and arvelexin) are found as phytoanticipins, i.e., they are constitutively produced in this species (Pedras and Adio, 2008).

CONCLUSION

In this paper we provided evidence for a specific pattern of ROS formation and accumulation in halophytic *Eutrema* in comparison to glycophytic *Arabidopsis*. In concert with that, a pronounced changes in the antioxidant system were revealed in *E. salsugineum*, such as a decreased activity of APX, an increased activity of GPX, an increased level of PrxQ, and the presence of thylakoid-bound forms of FeSOD and APX. All these cues allow for enhanced H_2O_2 signaling from chloroplasts of *E. salsugineum* already in the control conditions, at decreased level of oxidative damage in the same time. This signaling led to enhanced level of glucosinolates in *Eutrema* and decreased levels of stress hormones (SA and jasmonates). Salinity-stress evoked a strong up-regulation of all tested stress hormones in *A. thaliana* whereas in *E. salsugineum* only a stimulation in ethylene synthesis and ABA catabolism was noted. On this basis we hypothesize that H_2O_2 signaling is engaged in the halophytic species to modulate a hormonal responses in such a way to minimize the triggering of jasmonate pathway which leads to cell death.

AUTHOR CONTRIBUTIONS

MP performed analysis of antioxidants, staining and quantitation of H_2O_2, determination of glucosinolates, participated in TEM analysis and in preparation of the manuscript. MW measured MDA, participates in the analysis of antioxidants and ROS. IJ measured ROS with EPR. MK-K performed TEM analysis. PD and RV measured stress hormones, and RV participated in writing of the text. EN designed the experiment and wrote the manuscript. All authors have read and approved the manuscript.

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Patterns of ROS Accumulation in the Stigmas of Angiosperms and Visions into Their Multi-Functionality in Plant Reproduction

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Accumulation of reactive oxygen species (ROS) in the stigma of several plant species has been investigated. Four developmental stages (unopened flower buds, recently opened flowers, dehiscent anthers, and flowers after fertilization) were analyzed by confocal laser scanning microscopy using the ROS-specific probe $DCFH_2$-DA. In all plants scrutinized, the presence of ROS in the stigmas was detected at higher levels during those developmental phases considered "receptive" to pollen interaction. In addition, these molecules were also present at early (unopened flower) or later (post-fertilization) stages, by following differential patterns depending on the different species. The biological significance of the presence ROS may differ between these stages, including defense functions, signaling and senescence. Pollen-stigma signaling is likely involved in the different mechanisms of self-incompatibility in these plants. The study also register a general decrease in the presence of ROS in the stigmas upon pollination, when NO is supposedly produced in an active manner by pollen grains. Finally, the distribution of ROS in primitive Angiosperms of the genus *Magnolia* was determined. The production of such chemical species in these plants was several orders of magnitude higher than in the remaining species evoking a massive displacement toward the defense function. This might indicate that signaling functions of ROS/NO in the stigma evolved later, as fine tune likely involved in specialized interactions like self-incompatibility.

Keywords: stigma, reactive oxygen species, fluorescent probes, defense, pollen, development, self-incompatibility, signaling

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INTRODUCTION

The term reactive oxygen species (ROS) defines molecules derived from the metabolism of oxygen such as hydrogen peroxide or superoxide radical. In a similar way, reactive nitrogen species (RNS) includes reactive molecules derived from nitrogen metabolism, mainly the nitric oxide (NO). The presence of ROS and RNS must be balanced to maintain the correct cellular functions. When they are present in high concentrations, they may cause damage to the cell or even cell death. Hence, the role of the antioxidants is very important, in order to keep the correct balance of these species.

The study of both ROS and RNS in the Reproductive Biology of plants is an emerging discipline. These molecules are able to modulate and control the complex signaling cascades regulating the pollen–pistil interactions in Angiosperms. Several studies have been carried out in plants

considered as model like *Lilium longiflorum*, *Arabidopsis*, *Petunia* and a invasive plant in the United Kingdom such as *Senecio squalidus* (see review of Traverso et al., 2013). McInnis et al. (2006a,b) explored the amounts of ROS, particularly hydrogen peroxide, in stigmas and pollen from various different angiosperms by using the ROS probes $DCFH_2$-DA and TMB. They demonstrated that constitutive accumulation of ROS/H_2O_2 appears to be a feature of angiosperm stigmas, and discussed these results in terms of a possible role for stigmatic ROS/H_2O_2 and pollen-derived NO in pollen–stigma interactions and defense.

A former work by Zafra et al. (2010) was aimed to determine whether relevant ROS and RNS were present in the stigmatic surface and other reproductive tissues in the olive over different key developmental stages of the reproductive process. The olive tree is an important crop in Mediterranean countries. It is a dicotyledonous species, with some peculiarities in its reproductive organs. The presence of self-incompatible genotypes in this species has been described, as well as fertilization mainly allogamous (this means that a flower will be preferentially pollinated by pollen from a different cultivar; Mookerjee et al., 2005). The self-incompatibility system in this plant is of gametophytic type, although not well determined yet.

The main conclusions of this work were that both ROS and NO are produced in the olive reproductive organs in a stage- and tissue- specific manner, and that these chemicals may play different functions depending on these parameters. Thus, ROS and NO may foster defense functions against microbial or fungal attacks at the early flowering stages, whereas they also may determine the presence of a receptive phase in the stigma later on, or regulate pollen-pistil interaction. This work developed on olive also confirmed the emission of NO through the apertural regions of the pollen grains and the pollen tubes, the absence of these chemicals in the style or the ovary, and the decrease in the presence of ROS present in the stigma when NO was actively produced by pollen grains reaching this floral structure.

Some emerging literature has also described ROS and NO in the reproductive biology of other species like *Glycine max* (Li et al., 2012), *Corylus avellana* (Beltramo et al., 2012), *Helianthus* (Sharma and Bhatla, 2013), *Elaeocarpus hainanensis* and *Michelia alba* (Liu and Lin, 2013).

We have recently conducted analyses of ROS localization in species with different types of stigmas and systems of self-compatibility in order to reach general conclusions regarding the physiological roles of these products in plant reproduction.

MATERIALS AND METHODS

The conspicuous changes in the distribution and proportion of different ROS occurring in the reproductive tissues of the olive throughout flower development have been used as a model to compare this topic in other plants. Several stages (unopened flower buds, recently opened flowers, dehiscent anthers, and flowers after fertilization) have been studied by using $DCFH_2$-DA fluorophore and confocal laser scanning microscopy. The study was carried out in species with different types of stigmas and systems of compatibility.

Dissected floral buds or complete flowers were immersed in 50 μM $DCFH_2$-DA (Calbiochem) in MES (2- [N-morpholino]ethanesulfonic acid)-KCl buffer (5 μM KCl, 50 μM CaCl2, 10 mM MES, pH 6.15) for 10 min followed by a wash step in fresh buffer for 15 min, and then observed in a Nikon C1 confocal microscope using an Ar-488 laser source. Negative controls were treated with MES-KCl buffer only.

The intensity of the green fluorescence was quantified by using the Nikon EZ-C1 viewer (3.30) software. Both average and standard deviation were calculated after measurement of a minimum of nine images corresponding to three independent experiments.

RESULTS

Differences in the flower developmental patters of the selected species were in many cases obvious (**Figure 1A**), although a selection of similar stages was made based in the criteria described next. Flowers at stage 1 corresponded to unopened, generally green flower buds of the smaller size. Such flowers were usually dissected in order to make gynoecium available to the fluorochrome solution. At stage 2, flowers used were larger in size although still unopened; therefore removal of petals and sepals was frequently needed to make gynoecium prone to fluochrome incubation. Flowers at stage 3 were just opened and showed in most cases a significant change in the color of the petals. Gynoecia in these flowers were more easily accessible. Anthers in stage 3 contained pollen grains in most cases although anthers were not dehiscent yet. Stage 4 was characterized for anther dehiscence, with numerous pollen grains present on the stigma surface, whereas stage 5 corresponded to flowers already pollinized, displaying fallen corollas or degeneration of petals. For *Arabidopsis thaliana*, four stages were selected only, as this was the only species analyzed in which anther dehiscence took place before flower bud opening.

After treatment with $DCFH_2$-DA fluorophore, low-magnification observations detected green fluorescence in all samples analyzed. This fluorescence was in all cases restricted to the stigma, and absent from the remaining parts of the gynoecium (this is the style and the ovary), which only displayed red fluorescence assigned to autofluorescence. Such autofluorescence was comparable to that one present in the negative controls where the $DCFH_2$-DA fluorophore was omitted (**Figure 1B**). Anthers only showed fluorescence at dehiscence stages and during senescence. When higher magnification was used, the green fluorescence of the stigmas, resulting from $DCFH_2$-DA, was observed both in the stigmatic papillae and in the stigma exudate of those species where this later was present (results not shown).

Green fluorescence was analyzed and quantified in replica of the different experiments for each one of the species analyzed and the five different developmental stages. Results of quantification are represented in **Figure 1C** by using arbitrary

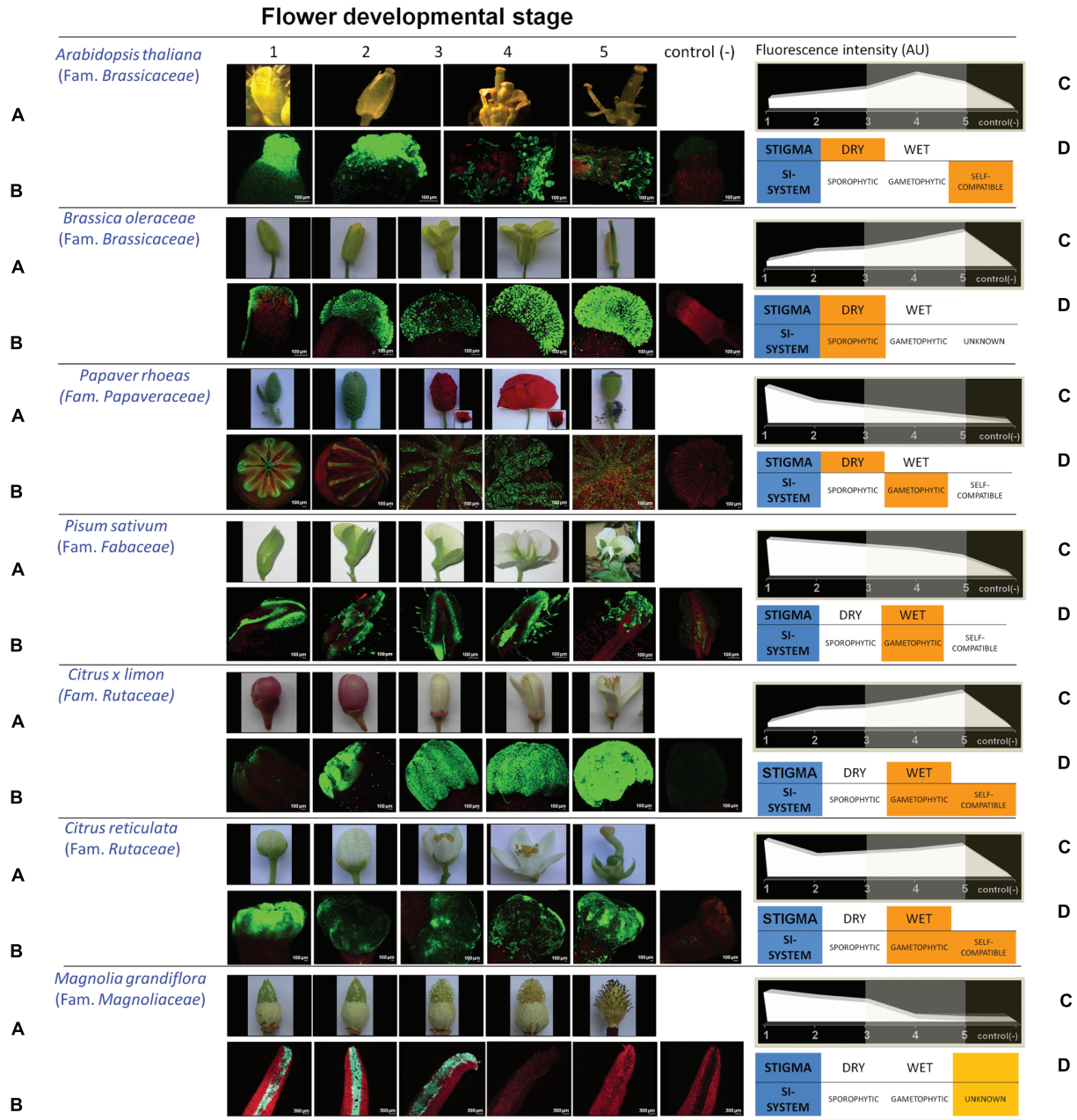

FIGURE 1 | Patterns of reactive oxygen species (ROS) distribution in different Angiosperm species. (A) Pictures showing flower developmental stages from different Angiosperm families. Five different stages were differentiated (four stages in *Arabidopsis thaliana*, displaying dehiscent anthers before flower bud opening). **(B)** 3-D reconstructions of the whole stigma surface from multiple optical sections. The presence of ROS is shown as a fluorescent signal (green) exclusively obtained in the range of 515–560 nm emission wavelengths, whereas tissues autofluorescence was recorded in red. Green fluorescence was extensively localized in the exudate (wet stigmas), as well as in stigmatic papillae. Identical settings were used for image capture in both control/test experiments in order to ensure reproducibility. **(C)** Developmental patterns resulting from fluorescence quantification (AU: arbitrary units). **(D)** Additional details as regards to type of stigma (dry, wet) and self-incompatibility system for each plant analyzed.

units of fluorescence. Different patterns of ROS accumulation were observed. In *A. thaliana*, *Papaver rhoeas*, *Pisum sativum* and *Camellia reticulata*, high levels of fluorescence were already observed at the very early flower buds (stage 1), whereas *Brassica oleracea* and *Citrus* × *lemon* displayed low levels of fluorescence at this same stage.

Stage 5, characterized by senescence of several flower organs after fecundation, also represented a differential step concerning fluorescence accumulation. In this case, high levels of fluorescence were observed in *B. oleracea*, *C. reticulate*, and *C* × *lemon*, whereas low level of stigmatic fluorescence appeared in the remaining species.

Relatively low fluorescence levels in comparison with the surrounding stages were observed in stages 3 and 4 for all species analyzed, coincidentally with the flower bud aperture and the putative presence of pollen grains over the stigma surface.

Reactive oxygen species detection was also performed in flowers of *Magnolia grandiflora* (Family *Magnoliaceae*), a representative species considered one of the most ancient lineages of present flowering plants. Flowers of this species are bisexual and display protogynous dichogamy in order to prevent self-incompatibility. First, female flowers open, and then a delayed second opening occurs after some time, with the flower functionally acting as a male (Losada et al., 2014 and references therein). Evidence indicates that stigma receptivity in plants of this family is brief, and has been reported to be finely coordinated with the secretion of AGPs (arabinogalactan proteins) in the stigma (Losada et al., 2014).

For this species, five developmental stages were defined as regard to female development (**Figure 1A**), including flower buds before and immediately after opening of green and white tepals (stages 1 and 2, respectively), recently opened tepals with green stigmas revealing curled tips (stage 3), colored stigmas with curled tips (stage 4), and senescent stigmas (brown) together with dehiscent anthers (stage 5).

After treatment with $DCFH_2$-DA fluorophore, medium-magnification observations detected green fluorescence over the stigmas surface only, mainly at stages 1−3. Green fluorescence was absent from the anthers and other areas of the flower at the stages analyzed, which showed red autofluorescence only, comparable to that one present in the negative controls where the $DCFH_2$-DA fluorophore was omitted (**Figure 1B**). Green fluorescence was analyzed and quantified. For this plant, the intensity of green fluorescence at the different replica was much higher than in the remaining species analyzed here, even after using the same experimental procedure and identical settings for image capture of the fluorescence under the same microscope equipment. Therefore, images were acquired using modified settings in order to prevent saturation of the microscope detectors. The resulting profile is displayed in **Figure 1C**, consisting in high levels of signal at the early stages (1 to 3), that quickly diminishes through stages 4 and 5 to nearly negligible levels.

DISCUSSION

Although a succinct number of plant species have been assayed to date, also through a limited number of developmental stages, several guidelines may arise from the present study. First, and coincidentally with the studies of McInnis et al. (2006a), Hiscock et al. (2007), Zafra et al. (2010), and Sharma and Bhatla (2013), our findings confirm that production of ROS is a prevalent feature of Angiosperm stigmas, detected in all species analyzed on this aspect so far.

A second feature demonstrated through the present and former studies consists in the limitation of the presence of detectable amounts of ROS to the tissues of the stigma surface, with the remaining floral organs lasting unlabelled (exception made to the anthers and pollen at dehiscent stages). One of the unique features of numerous stigmas in comparison with other floral organs is the presence of stigmatic exudate, extremely rich in nutrients, including sugars, lipids and proteins, which has been detected to accumulate ROS in many species (i.e., *P. sativum*, *Olea europaea* both *Citrus* species studied here, etc.; Serrano et al., 2008; Shakya and Bhatla, 2010; Suárez et al., 2012; Rejón et al., 2013). Moreover, we have also detected a massive presence of ROS in *M. grandiflora*, which also produces abundant secretions (Losada et al., 2014). Accumulation of ROS in the stigmatic exudate has been proposed by Hiscock et al. (2007) as a mechanism to protect against pathogen attack, on the same basis than flower nectars (Carter and Thornburg, 2000, 2004). However, and although this might represent a plausible explanation, we have detected ROS accumulation in species displaying stigmas of the dry-type like *A. thaliana*, *B. oleracea,* and *P. rhoeas*, therefore lacking of a significant stigma exudate. Plant species with dry and semi-dry stigmas have been described to harbor a thin pellicle which overlays the cuticle, often containing associated peroxidases (McInnis et al., 2006b). High-resolution microscopy studies would be necessary in order to assign the production of each one of the major ROS components to the tissue constituents of such stigmas.

As disclosed here, accumulation patterns for these chemicals through stigma development -a topic much less studied- offer a high level of variability among plant species. In spite of the still scarce number of stages and limited number of plants analyzed, different basic outlines have been observed. Apparently, patterns do not follow clear phylogenetic criteria, as different species from the same family do not share identical o similar models of ROS accumulation (e.g., *A. thaliana* and *B. oleracea*, both *Citrus* species studied here, and some other examples not shown -*O. europaea* and *Jasminum excelsior*-).

Sharp differences among species are visible just at the very early stages of flower development (stages 1-2), corresponding to unopened flowers. Zafra et al. (2010) discuss many of the physiological scenarios, which may concur at such stages, including the presence of high metabolic rates at the papillae and the surrounding tissues, and the defense issue mentioned above. What seems doubtful at these stages is the involvement of ROS in stigmatic receptivity and/or pollen-pistil signaling, as such stages do not physically involve pollen–pistil interaction. Then, why some plant species do not show high levels of ROS at early flowering stages? Discrepancies among species might therefore occur as the result of different rates of ROS production, for example because differences in the timing and intensity of the generated exudate, the growth rate of the floral tissues or metabolic rates (Liu and Lin, 2013).

Reactive oxygen species (mainly H_2O_2) scavenging has been widely correlated with launching of stigmatic receptivity, by means of the increased activity of enzymes like superoxide dismutases and peroxidases, even through the expression of new isoforms (McInnis et al., 2006b and cites therein; Sharma and Bhatla, 2013). Thus, tests for peroxidase activity have become the election method to measure pistil receptivity (Dafni and Motte Maués, 1998). Although not performed in the present study, such enzyme activities and ROS levels have been described

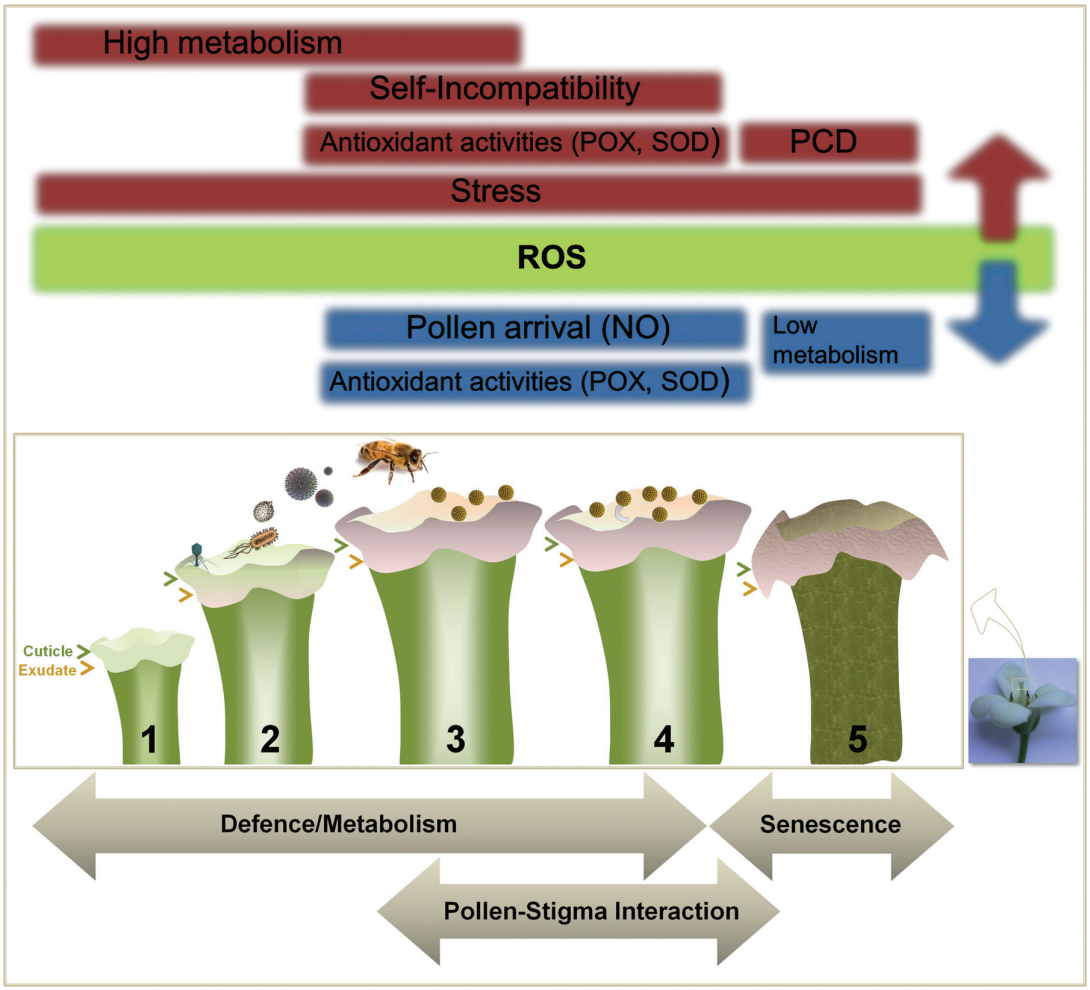

FIGURE 2 | Factors affecting ROS accumulation in Angiosperm stigma. Numerous intrinsic and extrinsic factors may increase (red) or reduce/scavenge (blue) basal levels of ROS produced through metabolism and present mainly on the stigma surface (green), modulating their accumulation and generating different profiles highly depending on the plant species. These factors can be grouped into three major categories: defense, pollen–pistil interaction and senescence.

to exhibit reverse trends during pollen–stigma interaction, the same tendency that occurs with regard to the production of NO by pollen grains reaching the stigmatic surface (McInnis et al., 2006a,b; Zafra et al., 2010; Sharma and Bhatla, 2013). In the majority of species tested here, ROS accumulation at stages 3 and 4, was overall lower than in the remaining stages. Although not particularly tested here, these observations are in good agreement with both situations: reaching of maximum stigmatic receptivity, and pistil interaction with pollen grains likely emitting NO.

Although the fine involvement of ROS in self-incompatibility mechanisms (particularly through the induction of PCD) is beginning to be undercover (Thomas and Franklin-Tong, 2004; Goldraij et al., 2006; Gao et al., 2010; Serrano et al., 2010, 2012; Allen et al., 2011; Wang and Zhang, 2011), no clear relationships between the differential patterns of ROS accumulation in the stigmas and the self-incompatibility mechanism applying for each species have been detected either. As an example, *B. oleracea* and *Citrus* × *lemon* display quite similar patterns of ROS accumulation, in spite of concealing different self-incompatibility systems (**Figure 1D**). On the contrary, species with similar self-incompatibility systems may differ broadly in their ROS-accumulating profiles (i.e., both *Citrus* species analyzed here, *P. rhoeas* and additional species not shown). Moreover, *A. thaliana* (self-compatible) and *P. rhoeas* (gametophytic SI) share rather similar profiles. Finally, *M. grandiflora* shows a nearly unique ROS-accumulating pattern. The prevalence of this type of profiles among other ancient species (either with protogynous dichogamy in order to prevent self-incompatibility, or other considered more evolved systems) is yet to be determined. The great contrast between the huge presence of ROS at the early stages and the near absence of these chemicals in *M. grandiflora* stigmas at those stages with pollen–stigma interactions might suggest that ROS function in these stigmas could be strongly unbalanced toward the defense function. Finely tuned signaling interactions among pollen and stigma, might be

then reduced or absent in this primigenius and singular plant, and appear later in evolution. However, these premises have to be further assessed.

An additional topic to be comprehensively examined is flower senescence, particularly stigma senescence as per stage 5 in the present study. Programmed cell death at this stage, triggered by the rise in ROS production, although frequent and widely described for petals (Rogers, 2012), shouldn't be considered a fully general trend in stigmas. Alternative patterns, with low production of ROS at stage 5 have been detected here in *Papaver* and *Magnolia*, again depending on the species analyzed. Finally, the role of ROS in the cellular events underlying in the gynoecium, like self-incompatibility events, pollen tube growth and directionability, and fertilization are beginning to be undercover (Heydlauff and Groß-Hardt, 2014).

CONCLUSION

The multifunctional nature of ROS, generated as a consequence of metabolism, involved in numerous stress, defense and signaling functions, and modulated through numerous enzymatic and non-enzymatic systems makes their presence a valuable marker of plant (flower) physiology. The presence of ROS in pollen and stigma (**Figure 2**) is likely influenced by a number of intrinsic (histochemical composition of the stigma, presence of exudate, cuticle, differential timing of floral development for each species, self-incompatibility mechanisms) and probably also extrinsic factors such as model of pollen dispersion, pollen viability, and stress (Zinn et al., 2010). The developmental changes observed involve many biochemical systems and molecular mechanisms, which both promote and counteract the increase of ROS (Cavaiuolo et al., 2013). This should be further analyzed in the different models for reproductive biology by means of the numerous tools available in order to obtain solid evidence supporting the hypotheses displayed here.

AUTHOR CONTRIBUTIONS

JA and SH planned the experimental approaches. AZ and JR collected the material and carried out the experimental work. AZ, JR, and JA performed imaging experiments. All authors contributed to the preparation, reviewed and approved the manuscript.

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Involvement of Polyamine Oxidase-Produced Hydrogen Peroxide during Coleorhiza-Limited Germination of Rice Seeds

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Seed germination is a complicated biological process that requires regulated enzymatic and non-enzymatic reactions. The action of polyamine oxidase (PAO) produces hydrogen peroxide (H_2O_2), which promotes dicot seed germination. However, whether and, if so, how PAOs regulate monocot seed germination via H_2O_2 production is unclear. Herein, we report that the coleorhiza is the main physical barrier to radicle protrusion during germination of rice seed (a monocot seed) and that it does so in a manner similar to that of dicot seed micropylar endosperm. We found that H_2O_2 specifically and steadily accumulated in the coleorhizae and radicles of germinating rice seeds and was accompanied by increased PAO activity as the germination percentage increased. These physiological indexes were strongly decreased in number by guazatine, a PAO inhibitor. We also identified 11 PAO homologs (*OsPAO1–11*) in the rice genome, which could be classified into four subfamilies (I, IIa, IIb, and III). The OsPAO genes in subfamilies I, IIa, and IIb (*OsPAO1–7*) encode PAOs, whereas those in subfamily III (*OsPAO8–11*) encode histone lysine-specific demethylases. *In silico*-characterized expression profiles of *OsPAO1–7* and those determined by qPCR revealed that *OsPAO5* is markedly upregulated in imbibed seeds compared with dry seeds and that its transcript accumulated to a higher level in embryos than in the endosperm. Moreover, its transcriptional abundance increased gradually during seed germination in water and was inhibited by 5 mM guazatine. Taken together, these results suggest that PAO-generated H_2O_2 is involved in coleorhiza-limited rice seed germination and that *OsPAO5* expression accounts for most PAO expression and activity during rice seed germination. These findings should facilitate further study of PAOs and provide valuable information for functional validation of these proteins during seed germination of monocot cereals.

Keywords: seed germination, polyamine oxidases, hydrogen peroxide, *Oryza sativa*, *OsPAO5*, gene expression, *in silico* analysis

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INTRODUCTION

Seed germination involves complex physiological and biochemical processes, e.g., signal transduction and gene expression regulation (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Gomes and Garcia, 2013; He and Yang, 2013; Han and Yang, 2015). For dicot seeds, e.g., Arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*), cress (*Lepidium sativum*)

and tobacco (*Nicotiana tabacum*), their micropylar endosperm (usually denoted the endosperm cap) is mechanically strong and acts as a physical barrier to the completion of germination (Leubner-Metzger and Meins, 2000; Nonogaki et al., 2000; Müller et al., 2006; Iglesias-Fernandez and Matilla, 2010; Nonogaki, 2014). In monocot seeds, particularly those of cereals, e.g., barley (*Hordeum vulgare*), rice (*Oryza sativa*), and purple false brome (*Brachypodium distachyon*), the coleorhiza, which is a non-vascularized multicellular embryonic tissue covering the seminal seed root, is believed to regulate emergence of the radicle during germination (Millar et al., 2006; Gonzalez-Calle et al., 2015). It has been assumed that dicot seed germination is controlled by the mechanical force of the imbibed, elongating radicle on the endosperm cap and by inherent cap weakening driven by enzymatic (i.e., endo-β-1,4-mannanases and pectin methylesterases) and non-enzymatic reactions [e.g., those involving reactive oxygen species (ROS); Nonogaki et al., 2010; Zhang et al., 2014; Scheler et al., 2015; Chen et al., 2016]. Given the physical and chemical similarities of dicot and monocot seed structures, logically similar enzymatic and non-enzymatic mechanisms would be required during monocot seed germination.

Previous studies have shown that ROS, e.g., the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$), function as positive and negative signaling molecules during seed germination (D'Autreaux and Toledano, 2007; Tripathy and OelMüller, 2012; Gomes and Garcia, 2013). The involvement of ROS in seeds, e.g., endosperm weakening, mobilization of seed reserves, protection against pathogens, and programmed cell death, is well-known (El-Maarouf-Bouteau and Bailly, 2008; Gomes and Garcia, 2013). H_2O_2 serves as a molecular 'hub' during ROS-mediated signaling in plants; specifically, in seeds it is important for cell wall loosening, which is necessary for seed germination (Quan et al., 2008; Gomes and Garcia, 2013; Wojtyla et al., 2016). The ROS with the greatest reactivity and shortest life span is $^{\cdot}OH$, which is formed from O_2^- and H_2O_2 in the apoplast by the action of cell wall peroxidases and can directly degrade cell wall polysaccharides, thereby loosening the cell wall (Schweikert et al., 2000; Müller et al., 2009).

Polyamine (PA) catabolism is an important pathway for H_2O_2 generation. Polyamine oxidases (PAOs) generate H_2O_2 by oxidative degradation of the PAs putrescine (Put), spermidine (Spd), and spermine (Spm). PAs are aliphatic amines of relatively small molecular mass involved in various physiological processes in plants, e.g., growth, development, and stress responses (Alcázar et al., 2010; Mattoo et al., 2010). The PAOs, copper-dependent diamine oxidases (EC 1.4.3.6), and flavin adenine dinucleotide-associated PAOs (EC 1.5.3.11) catalyze oxidation of deaminated moieties at primary and secondary amino groups while generating H_2O_2 as a product (Kusano et al., 2008; Moschou et al., 2012; Planas-Portell et al., 2013). Based on the chemical structures of their reaction products, PAOs are classified as: (i) those responsible for terminal catabolism of PAs, during which the carbon on the endo-side of the N_4-nitrogens of Spd and Spm is oxidized-these PAOs are only found in plants (Cervelli et al., 2006; Moschou et al., 2008b; Liu et al., 2014a); (ii) those responsible for back-conversion of PAs by oxidizing the carbon on the exo-side of the secondary amino of N_1-acetylderivatives in animals and non-acetylated PAs in plants (Tavladoraki et al., 2006; Kamada-Nobusada et al., 2008; Moschou et al., 2008b; Ono et al., 2012); (iii) those that contain a PAO domain but do not deaminate PAs; instead they demethylate histone H3K4 in animals and plants (Shi et al., 2004; Spedaletti et al., 2008; Mosammaparast and Shi, 2010; Luo et al., 2014; Prakash et al., 2014).

The biological significance and physiological functions of PAOs from several organisms have been characterized. For example, the PAOs in the monocots maize (*Zea mays*; *ZmPAO1*), barley (*HvPAO1* and *HvPAO2*), and rice (*OsPAO7*) are involved in the terminal catabolism of PAs, and they oxidize the carbon at the endo-side of the N_4 of Spm and Spd to produce *N*-(3-aminopropyl)-4-aminobutanal and 4-aminobutanal, respectively, 1,3-diaminopropane, and H_2O_2 (Tavladoraki et al., 1998; Cona et al., 2005; Cervelli et al., 2006; Liu et al., 2014a). The five PAOs in *Arabidopsis* (*AtPAO1–5*) and four of seven PAOs in rice (*OsPAO1*, *OsPAO3*, *OsPAO4*, and *OsPAO5*) catalyze the back conversion of Spm (or T-Spm) to Spd and/or Put in a manner similar to that of animal PAOs/SMOs (Kamada-Nobusada et al., 2008; Moschou et al., 2008c; Takahashi et al., 2010; Fincato et al., 2011; Liu et al., 2014b). Moreover, the aforementioned PAOs are found in different subcellular locations, during different developmental stages, or have different tissue-specific expression profiles. For example, *ZmPAO1*, *HvPAO1/2*, and *OsPAO7*, involved in terminal catabolism of PAs, are located at the edge of the plant cell although *HvPAO1/2* and *OsPAO7* expression is greatest in ear organs, sterile spikelets, and anthers (Tavladoraki et al., 1998; Cona et al., 2005; Cervelli et al., 2006; Ono et al., 2012; Liu et al., 2014a). In contrast, *AtPAO1–5* and *OsPAO1/3–5*, involved in back-conversion of Spm and T-Spm, are present in the cytoplasm and peroxisomes, with the *OsPAO3–5* transcription levels greatest in 2-weeks-old seedlings and the *OsPAO1* expression lowest (Fincato et al., 2011; Ono et al., 2012). In addition, the most abundant transcripts of *AtPAO1/2/3/5* are in flowers, whereas the highest level of *AtPAO4* expression is found in young seedlings, particular in their roots (Takahashi et al., 2010). Furthermore, *AtPAO4* deficiency is induced by alterations in the expression of genes related to drought stress response and flavonoid biosynthesis (Kamada-Nobusada et al., 2008). Interestingly, the third group of PAOs, the *Arabidopsis* and rice homologs of human lysine-specific demethylases, regulate flowering time and, for *Arabidopsis* seed dormancy, by demethylation of histone H3K4 (Shi et al., 2004; Jiang et al., 2007; Spedaletti et al., 2008; Mosammaparast and Shi, 2010; Luo et al., 2014; Shafiq et al., 2014; Zhao et al., 2015).

Although these studies on *Arabidopsis*, maize, barley, and rice PAOs have led to an understanding of their biochemical properties and physiological functions, characterization of PAO functions during rice seed germination has not been undertaken. Recent work has shown that ROS may have a regulatory role during the life stages of seeds, e.g., germination and release

from dormancy (Nonogaki, 2014). Therefore, because PAOs generate H_2O_2, they may be involved in seed germination via PA catabolic pathways. For the study reported herein, we performed a comprehensive evaluation of the role(s) played by PAOs during germination of rice seeds. Our study included characterizing the morphology of the germinating seeds, a histochemical analysis, quantification of ROS accumulation, measurement of PAO activity, and assessment of PAO gene expression profiles. The results should increase our understanding of the involvement of rice PAOs and their reaction product H_2O_2 in coleorhiza-limited seed germination and allow for further studies of the physiological role(s) of the PA catabolic pathways in plants.

MATERIALS AND METHODS

Non-plant Materials

Guazatine, *N*,*N*′-dimethylthiourea (DMTU), nitroblue tetrazolium (NBT), 3, 3-diaminobenzidine hydrochloride (DAB), 3,3′,5,5′-tetramethylbenzidine (TMB), Spm, Spd, Put, 4-aminoantipyrine, *N*,*N*′-dimethylaniline, and horseradish peroxidase were purchased from Sigma-Aldrich. Water used was always doubly distilled.

Plant Materials and Seed Germination

Rice seeds (*O. sativa* ssp. *japonica* cv. Nipponbare) with the glume removed were placed into a transparent plastic germination box (12 cm × 12 cm × 6 cm) containing two layers of filter paper soaked in water; 5 mM DMTU; 10 mM H_2O_2; 5 mM guazatine; 5 mM DMTU plus 10 mM H_2O_2; or 5 mM guazatine plus 10 mM H_2O_2 (20 mL each). The seeds were incubated in a growth chamber at 28 ± 1°C under a 16-h light/8-h dark photocycle (10,000 lux). Seeds with protruding radicles were regarded as having finished germination and were counted at 6-h intervals from 12 to 48 h. The number of germinated seeds at each time point was converted to a percentage, and the mean value ± SE of three biological replicates of 100 seeds each was calculated. Seeds were photographed using the stereomicroscope (SteREO Lumar V12, Zeiss, Germany).

Histochemical Localization and Quantification of O_2^- and H_2O_2

We used NBT and DAB, respectively, to stain seeds for O_2^- and H_2O_2 as described (Zhang et al., 2014; Chen et al., 2016). After rice seeds had imbibed water or 5 mM guazatine for 3, 6, 12, 24, or 48 h, five whole seeds and five half granule seeds containing the embryos were removed and incubated with 1 mM NBT in 10 mM Tris-HCl (pH 7.0), or 1 mg/mL DAB (pH 3.8) at room temperature for 30 min, then washed with double-distilled water, and photographed using the stereomicroscope (SteREO Lumar V12, Zeiss, Germany).

The rate of O_2^- production (nmol $O_2^-\cdot min^{-1}\cdot g^{-1}$ FW) and H_2O_2 concentration ($\mu mol\cdot g^{-1}$ FW) were spectrophotometrically measured as described [fresh weight (FW); Zhang et al., 2014; Chen et al., 2016]. Thirty embryos at each aforementioned imbibition time points were used for each type of measurement, and the mean value ± SE of three biological replicates were calculated.

Histochemical Detection of Peroxidase Activity

POD activity was detected histochemically by TMB staining as described (Zhang et al., 2014; Chen et al., 2016). Rice seeds were imbibed in water or 5 mM guazatine for the aforementioned five times. Five whole seeds and five half granule seeds containing their embryos were incubated in 0.2% (w/v) TMB, 1 mM H_2O_2, 20 mM phosphate (pH 6.5) at room temperature for 30 min, then washed with water, and photographed using the stereomicroscope (SteREO Lumar V12, Zeiss, Germany).

PAO Activity Assay

Embryos (0.2 g) from whole seeds imbibed for the aforementioned five times were extracted and immediately ground in a TissueLyser-24 (Shanghai Jingxin Industrial Development, Co., Ltd, China) at 4°C in 1.0 mL 0.1 mol/L sodium phosphate (pH 6.5). The homogenates were centrifuged at 10,000 × *g* and 4°C for 20 min. The supernatants were individually transferred into new tubes and centrifuged again at 5,000 × *g* and 4°C for 5 min. The second set of supernatants were assayed for PAO activity. To determine the optimal substrate and wavelength for PAO activity measurements, first the oxidation of Spm, Spd, or Put was observed after horseradish peroxidase oxidation of 4-aminoantipyrine and *N*,*N*′-dimethylaniline monitored between 300 and 800 nm (Su et al., 2006; Tavladoraki et al., 2006; Liu et al., 2014a). The reaction solutions (3.0 mL) each contained 2.5 mL 100 mM sodium phosphate (pH 6.5), 100 mM 4-aminoantipyrine, 1 mM *N*,*N*′-dimethylaniline, 0.1 mL horseradish peroxidase (250 U/mL), 0.2 mL of a crude enzyme extract and 0.2 mL of a substrate (20 mmol/L Spm, Spd, or Put). Assays were initiated by addition of a substrate and incubated at 30°C for 30 min. A_{515} was measured using a Multiskan Spectrum spectrophotometer (Varioskan Flash, Thermo, USA). A 0.01 change in the A_{515} was defined as one enzyme activity unit.

Identification and Phylogenetic Analysis of a PAO Gene Family

The latest non-redundant set of protein sequences for the monocot, *O. sativa*, and eudicot, *A. thaliana*, were retrieved from the Rice Annotation Project Database[1] and the *Arabidopsis* Information Resource (TAIR v10.0[2]), respectively. The sequences were incorporated into an in-house database and the procedures described in Li et al. (2014), Chang et al. (2016) were used to identify the rice and *Arabidopsis* PAO homologs, with the one difference that the family specific amino oxidase domain (PF01593) HMM profile was used in the HMM search. Then, after aligning the amino oxidase domain sequences of

[1]http://rapdb.dna.affrc.go.jp/index.html
[2]http://www.arabidopsis.org/

the identified PAO proteins, they were used to construct a phylogenetic tree as described in Li et al. (2014), Chang et al. (2016).

In silico Expression Profiles (Heat Maps) and Quantitative Real-Time PCR (qPCR) of PAO Homologs

We used the Os_51k microarray data in the Genevestigator V3 database to analyze the expression profiles of rice PAO genes, by constructing heat maps from the data sets (Hruz et al., 2008).

To characterize the expression profiles of OsPAO genes by qPCR, 30 embryos from seeds incubated in water or 5 mM guazatine for the aforementioned five imbibition times were extracted and immediately frozen at −80°C. Total RNA was isolated using Column Plant RNAout 2.0 kit reagents (TIANDZ, China) according to the manufacturer's instructions, and qPCR was performed as described (Li et al., 2014; Chang et al., 2016). The gene-specific primers used (Supplementary Table S3) were designed to avoid conserved regions, introns, and an exon–exon junction. *OsGAPDH1* (RAP-DB ID: Os02g0601300) expression served as the internal control. Mean value ± SE of three biological replicates were calculated.

Statistical Analysis

Data are presented as the mean ± SE of three replicates. One-way analysis of variance was used to compare mean values, and when significant, differences between individual means were compared with the Fisher's least-significant difference test. Student's *t*-test were conducted to evaluated variances in the expression levels of *OsPAO1–7*.

RESULTS

Rice Seed Germination is Promoted by Exogenous and PAO-Produced Endogenous H_2O_2, But Is Inhibited by DMTU and Guazatine

To determine whether PAO production of H_2O_2 promotes germination of rice seeds, we characterized the morphology and percentage of germinating seeds that had been imbibed in only water or in aqueous solutions containing exogenously added H_2O_2, DMTU (a scavenger for H_2O_2), guazatine (a competitive inhibitor of PAOs), H_2O_2 and DMTU, or H_2O_2 and guazatine at various times (**Figure 1**). The first seeds to complete germination in water did so by 12 h [**Figures 1A(top),B,C**]; 50% of the seeds incubated in water completed germination by 30 h, and 84% within 48 h (**Figures 1B,C**). Germination was promoted by 10 mM H_2O_2 but inhibited by 5 mM DMTU (**Figure 1B**). When the seeds were imbibed in 5 mM DMTU plus 10 mM H_2O_2, the germination percentage was always less than that for seeds germinated in water alone but greater than that for seed germinated in 5 mM DMTU (**Figure 1B**). Notably, 5 mM guazatine did not introduce a lag period before germination was observable, but reduced the germination percentage and inhibited the growth of the coleoptile and radicle (**Figures 1A,C**). When seeds were imbibed in 5 mM guazatine plus 10 mM H_2O_2, the extent of germination was completely recovered at each time point (**Figure 1C**). These results demonstrate that a PAO(s) may promote rice seed germination by producing H_2O_2 via oxidative degradation of PAs.

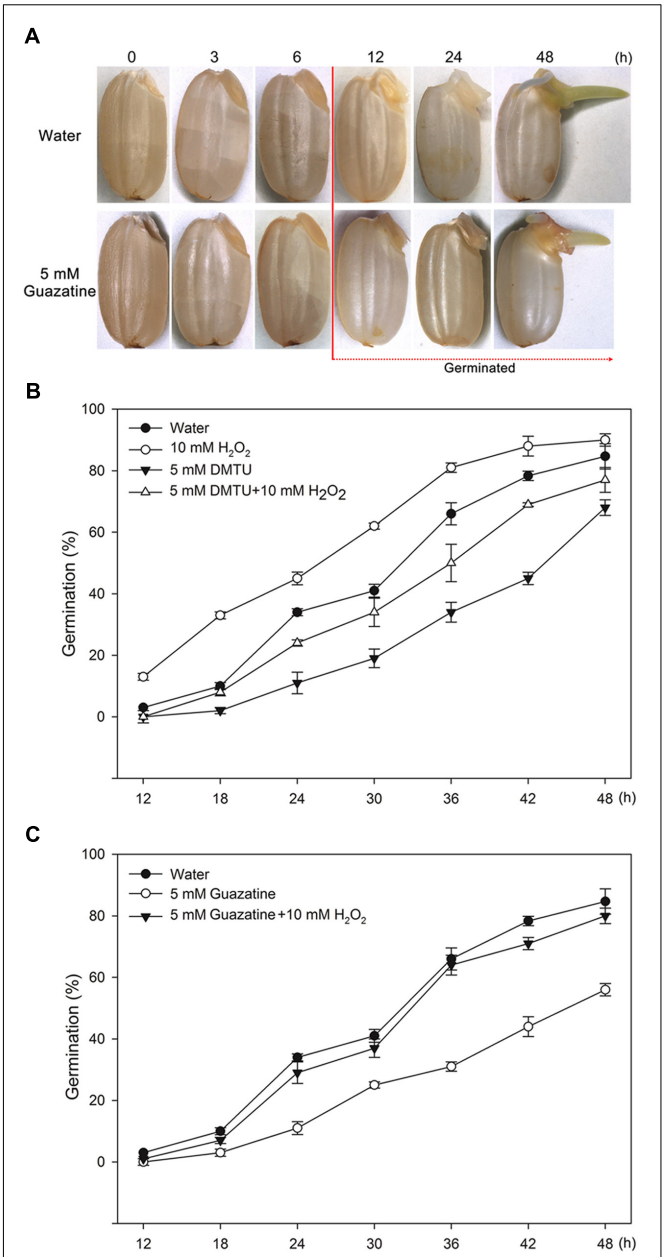

FIGURE 1 | Morphologies and germination time courses of rice seeds. **(A)** Morphologies of rice seeds imbibed in (top) water or in (bottom) 5 mM guazatine. Germination time courses for rice seeds imbibed in **(B)** water, 10 mM H_2O_2, 5 mM DMTU, 5 mM DMTU plus 10 mM H_2O_2, 5 mM guazatine, or **(C)** water, 5 mM guazatine or 5 mM guazatine plus 10 mM H_2O_2. Germination number was scored every 6 h for 48 h, and the results are presented as the cumulative germination percentages. Data are the mean ± SE of three biological replicates of 100 seeds each.

ROS are Produced and Accumulate in the Embryo and Aleurone Layer of the Rice Seed upon Imbibition in Water, But This Process Is Partially Inhibited by Guazatine

To characterize the distribution of ROS in germinating seeds, the presence of H_2O_2, O_2^-, and POD activity (an indirect measure of the production and accumulation of $^{\cdot}OH$) were detected, respectively, by DAB, NBT, and TMB staining of the aleurone layer and embryo. H_2O_2, O_2^-, and $^{\cdot}OH$ accumulated throughout the time course of the germination period. The embryo and aleurone layer were somewhat stained by all three stains, whereas the starchy endosperm was not (**Figures 2A,C,E,F**).

When seeds imbibed only in water, the whole seed was stained by DAB, with the embryo, especially its radicle, coleorhiza, and coleoptile, most strongly stained (**Figure 2A**). Staining of the embryo increased as the imbibition time increased, except that the coleoptile was only faintly stained at 48 h. Conversely, for seeds imbibed in 5 mM guazatine, their embryos, especially their radicles, were stained to a lesser extent (**Figure 2A**). The H_2O_2 content in the embryos was quantified spectrophotometrically (**Figure 2B**), which showed that when seeds were imbibed in water, the H_2O_2 content in the embryo increased throughout the imbibition time. However, when seeds were imbibed in 5 mM guazatine, their H_2O_2 content increased more slowly and its concentration in the embryos was greatly reduced compared with that for embryos imbibed in water. These results agree with those of the histochemical staining (**Figure 2A**), indicating that guazatine significantly inhibits H_2O_2 production in the embryos of germinating rice seeds.

Production and accumulation of O_2^- were also investigated by NBT staining of the embryos of the rice seeds imbibed in water or 5 mM guazatine. When the seeds were imbibed in water, their embryos were stained only after 12 h, and moreover, their coleorhiza, coleoptile, and radicle stained strongly after 12 h. As was found for water imbibition, the embryos were not initially stained when the seeds were imbibed in 5 mM guazatine, but were stained after 12 h (**Figure 2C**). The rate of O_2^- production in the embryos was also quantified spectrophotometrically (**Figure 2D**) and found to increase slowly before 6 h of water imbibition, increase rapidly thereafter, and be maintained between 24 and 48 h. When seeds were imbibed in 5 mM guazatine, however, the rate of O_2^- production was not significantly different to that found for water imbibition throughout most of the experiment. Therefore, unlike H_2O_2 production, O_2^- production was not suppressed by guazatine.

Because POD catalysis produces $^{\cdot}OH$ (Schopfer et al., 2001; Liszkay et al., 2004; Ren et al., 2008; Gonzalez-Calle et al., 2015), we assessed the POD activity in rice seeds that had been imbibed in water or in 5 mM guazatine by TMB staining (**Figure 2E**). Whole seeds imbibed in water or guazatine were completely TMB stained after 12 h. The intensity of the TMB stain in the embryo increased throughout the imbibition time in water and guazatine. For seeds imbibed in 5 mM guazatine, the intensity of the TMB staining (especially in the embryo) was less than that for those imbibed in water, indicating that guazatine probably reduced POD activity in the rice seeds.

Activity of Rice PAO(s), for Which Spm Is the Optimal Substrate, Increased Gradually in Embryos of Rice Seeds upon Water Imbibition and Was Intensely Inhibited by Guazatine Imbibition

A crude PAO embryo extract was assessed for PAO activity. Initially, we determined the substrate specificities and optimum absorption peak for the assay with Spm, Spd, and Put as substrates. When assayed, the crude extract had an absorbance peak centered at 515 nm (**Figure 3A**), a finding similar to that for PAOs from the lateral root of soybean (peak maximum at 555 nm; Su et al., 2006). The maximum activity was obtained for Spm as the substrate (**Figure 3A**). Therefore, Spm was used as the substrate for the time course experiment described below.

We determined the PAO activity in embryos of rice seeds during germination in water and in guazatine (**Figure 3B**). PAO activity in dry seeds (0 time of imbibition) was minimal, but increased in the embryos as the time of imbibition in water increased. Furthermore, by the end of the experiment (48 h) the mean radicle length was ∼1 cm (**Figure 1A**). The data indicate that PAO activity may be important for seed germination and radicle elongation. When the seeds were imbibed in 5 mM guazatine, PAO activity in the embryo decreased strongly, and it was significantly less than in dry seeds, suggesting that guazatine specifically reduced PAO activity in the rice seed during germination.

Phylogenetic Analysis of PAO Gene Family Indicated 11 PAO Homologs in Rice Were Classified into Four Well-Conserved Subfamilies with Distinct Subcellular Locations, Domain Organizations, and Diversified Functions

To characterize the phylogenetic relationship among rice and *Arabidopsis* PAO family genes, first a hidden Markov model search was performed to find the sequences related to the family specific amine_oxidase domains (PF01593), and a total of 11 rice and 9 *Arabidopsis* PAO homologs were identified (Supplementary Table S1). Then, an unrooted maximum-likelihood (ML) phylogenetic tree (**Figure 4**) was constructed using these sequences (Supplementary Figure S1). According to the topology and the deep-duplication nodes of the tree, these PAOs can be classified into the four well-known and conserved subfamilies (I, IIa, IIb, and III; **Figure 4A**) with statistical confidence. In addition to the typical amino_oxidase domain found in these proteins, subfamily III also contain a SWIRM (PF04433) domain upstream of the amino oxidase domain (**Figure 4B**). Notably, the subfamily III proteins are not PAOs, but histone lysine-specific demethylases, which catalyze the demethylation of H3K4 histone lysine residues via an FAD-dependent oxidation. These demethylases regulate plant growth and developmental processes, e.g., flowering time and seed

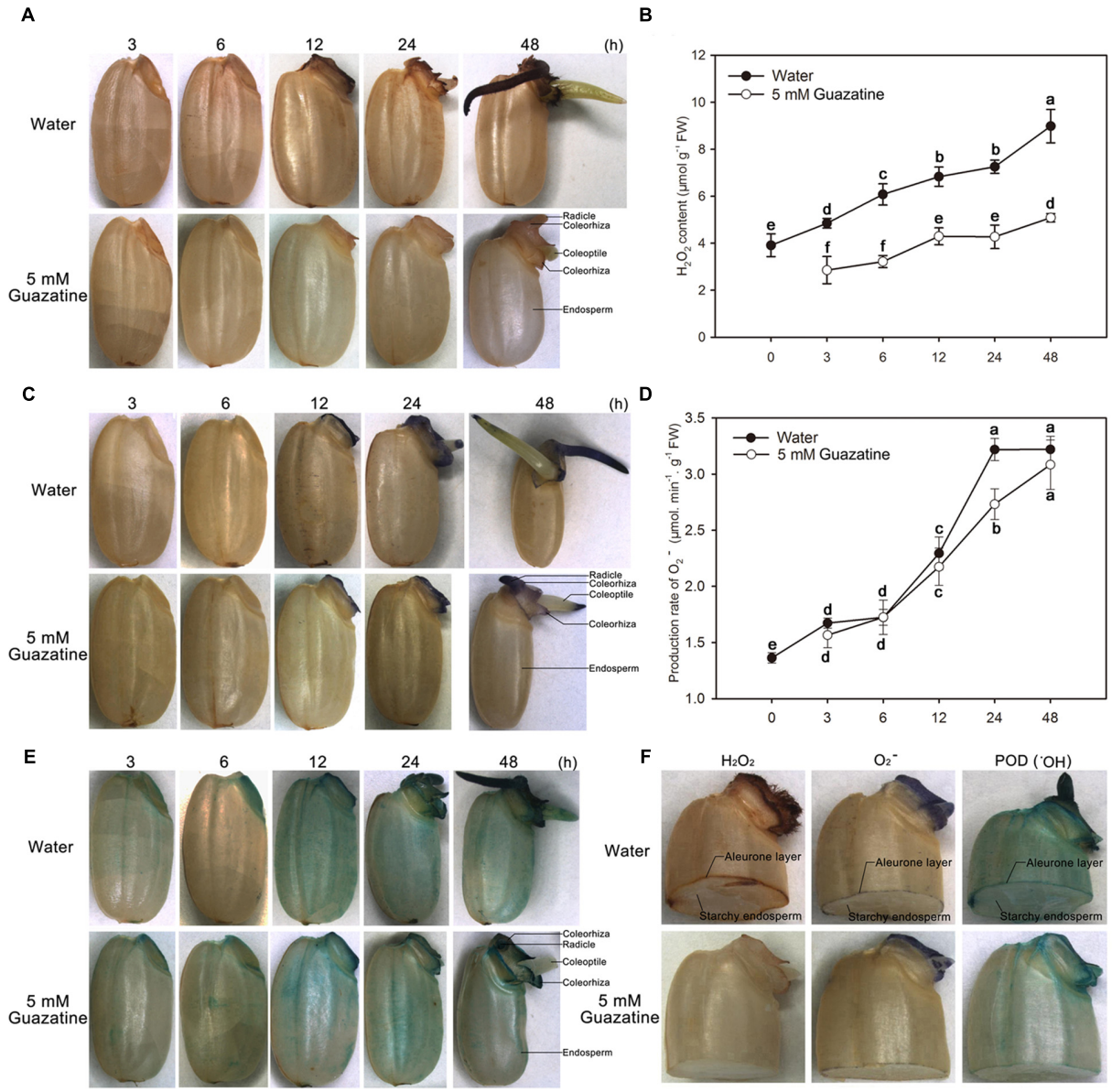

FIGURE 2 | Histochemical staining and quantification of H_2O_2 and O_2^- content, and peroxidase activity during germination of rice seeds in water or guazatine. Histochemical staining for the location of **(A)** H_2O_2 **(C)** O_2^-, and **(E)** peroxidase activity in whole rice seeds and in **(F)** embryos of half granule seeds after imbibition of water or 5 mM guazatine. Quantitative determination of **(B)** H_2O_2 content and **(D)** O_2^- production in seeds imbibed in water or in 5 mM guazatine. Data are the mean ± SE of three biological replicates of 30 embryos (~0.1 g total). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Fisher's least significant difference test. FW, fresh weight.

dormancy (Spedaletti et al., 2008; Luo et al., 2014; Prakash et al., 2014; Zhao et al., 2015).

Furthermore, we identified the subcellular locations of these proteins with the use of the crop Proteins with Annotated Locations database[3] and SubCellular Proteomic database[4], respectively (Supplementary Table S1). The classifications, locations, and functions (Tavladoraki et al., 1998; Cervelli et al., 2006; Jiang et al., 2007; Takahashi et al., 2010; Fincato et al., 2012; Ono et al., 2012; Liu et al., 2014a,b; Luo et al., 2014) of the proteins are summarized in **Figure 4B**. The subfamily I, IIa, and IIb PAOs are FAD-dependent amine oxidases and catalyze the catabolism of PAs. The subfamily I PAOs catalyze the final step in PA catabolism and are located extracellularly in the apoplast, whereas the subfamily IIa and IIb PAOs catalyze the back conversion of PAs and are located in the peroxisome and cytoplasm, respectively. However, the subfamily III proteins, although they have a typical amino oxidase domain, are histone lysine-specific

[3]http://croppal.org/

[4]http://suba.plantenergy.uwa.edu.au/

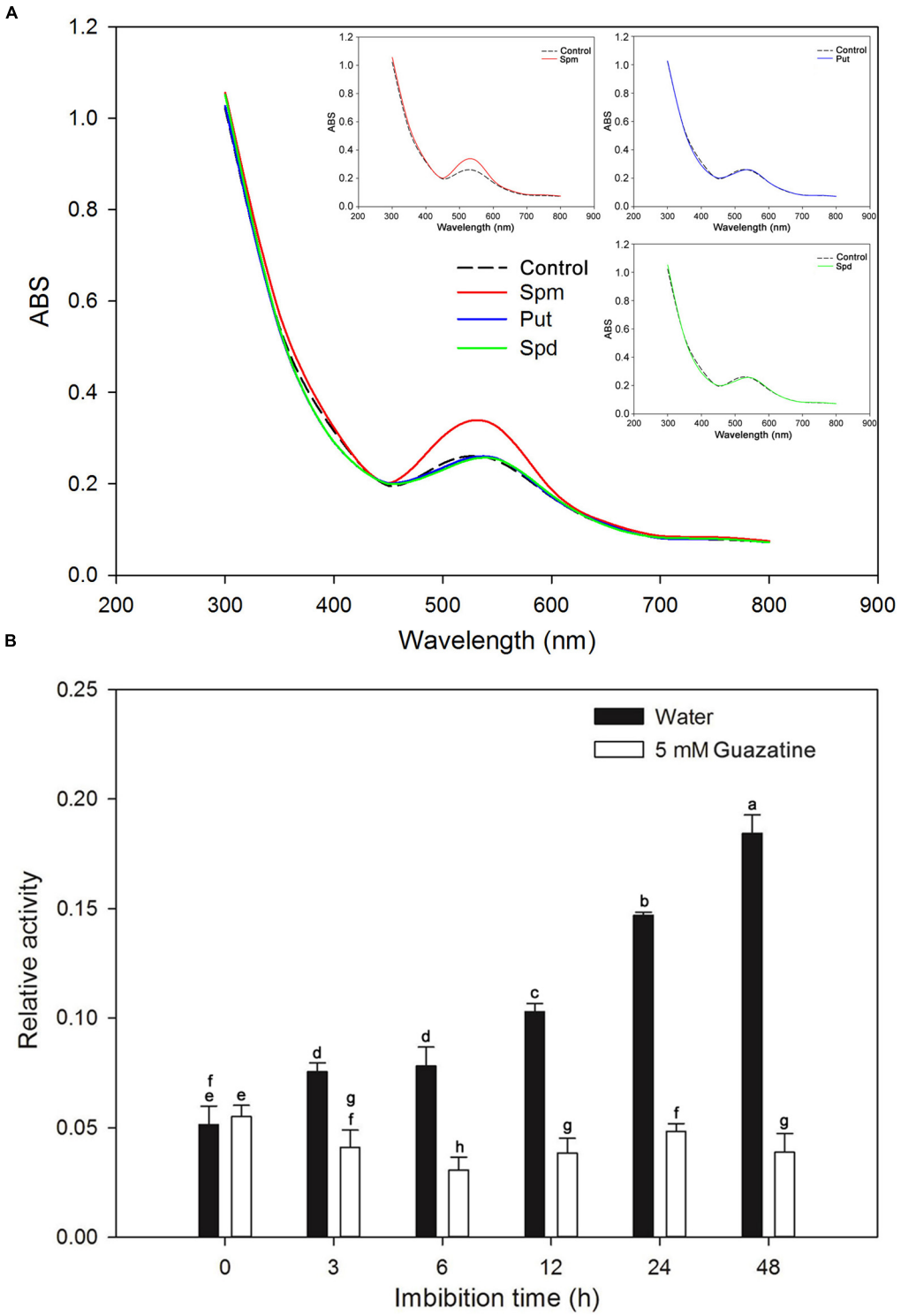

FIGURE 3 | Identification of the optimal substrate and absorbance peak for crude PAO activity and measurement of PAO activity during rice seed germination. (A) Substrate specificity and absorbance spectra found for the crude PAO activity in rice seeds after imbibed in water for 12 h. **(B)** Crude PAO activity in rice seeds, imbibed in water or in 5 mM guazatine at 3, 6, 12, 24, and 48 h, was determined using Spm as the substrate. Data are the mean ± SE of three biological replicates of 60 embryos (~0.2 g total). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Fisher's least significant difference test.

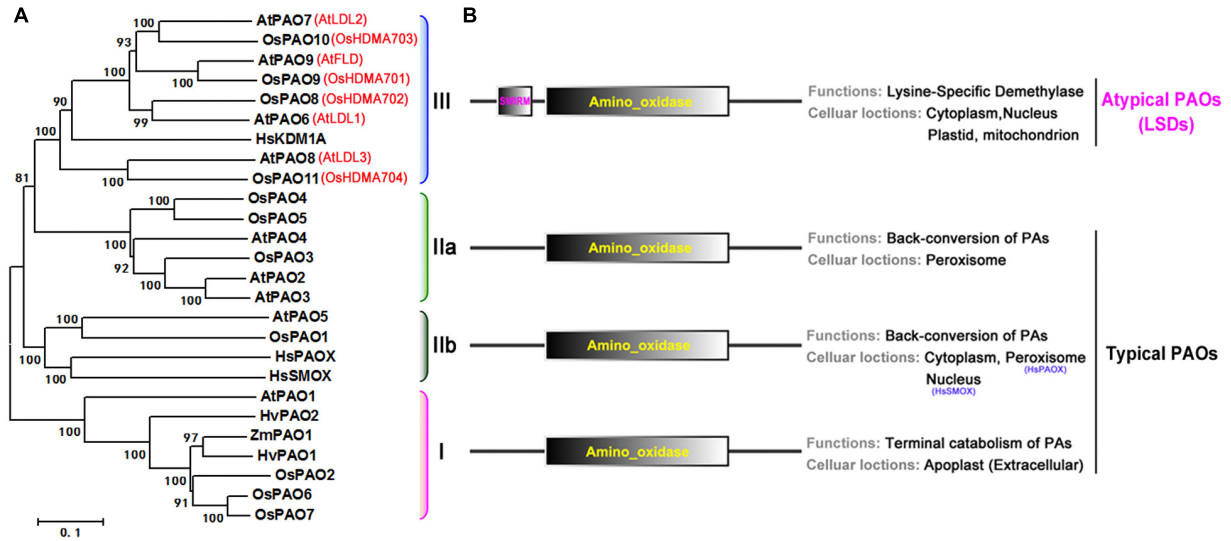

FIGURE 4 | Phylogenetic tree, predicted locations and functions of PAO protein family. (A) Unrooted maximum-likelihood phylogenetic tree for rice and *Arabidopsis* PAO protein family constructed based on an amino acid sequence alignment of their amino_oxidase domains. Human, maize, and barley sequences are included. HsPAOX (*Homo sapiens*, ENSP00000278060), HsSMOX (*H. sapiens*, ENSP00000307252), HsKDMA (*H. sapiens*, ENSP00000383042), ZmPAO1 (*Zea mays*, NM_001111636), HvPAO1/2 (*Hordeum vulgare*, AJ298131 and AJ298132). The associated bootstrap values from 1000 replications are given at their nodes, and the branch lengths are drawn to scale. The subfamily members are bracketed by color, and their subfamily numbers, I, IIa, IIb, and III, are shown to the right of the tree. **(B)** Domain organizations (left), and predicted functions and locations (right).

H3K4 demethylases, and are found in many different organelles such as the nucleus, cytoplasm, plastid, and mitochondria. Thus, given their domain organizations and catalytic activities, these proteins can be categorized as typical PAOs (subfamilies I, IIa, and IIb, OsPAO1–7 and AtPAO1–5) and atypical PAOs or lysine-specific demethylases (subfamily III, OsPAO8–11 and AtPAO6–9).

The Expression Profiles of *OsPAO1–7* Differ Significantly during Germinations, and the Transcript Level of *OsPAO5* Parallels that of PAO Activity and Change in H_2O_2 Content in the Embryo during Germination

We examined the expression patterns of *OsPAO1/3–5/7*, by displaying the rice microarray data from the Genevestigator database as heat maps (**Figure 5A**) and found significant differences in the expression of these genes during germination as opposed to dry seeds. Moreover, the expression levels of these genes were distinctly different for the embryo and endosperm during germination. The *OsPAO5* expression levels were markedly upregulated (1.48–8.76 fold) in germinating seeds compared with those in dry seeds, whereas other *OsPAO* expression levels were not obviously different (**Figure 5A**; Supplementary Table S2).

To further assess the expression profiles of *OsPAO1–7*, qPCR was performed, and the results (**Figure 5B**) are consistent with the expression patterns from the microarray data. Transcription of *OsPAO5* was markedly upregulated in the embryos of germinating seeds compared with that in dry seeds, and the expression of *OsPAO6/7* was significantly downregulated, whereas the expression levels of the other genes were not obviously changed.

The expression profile of *OsPAO5* was then examined throughout the course of seed germination (at 0, 3, 6, 12, 24, and 48 h) by qPCR (**Figure 5C**). For seeds imbibed in water, the *OsPAO5* expression in the embryos progressively increased reaching its greatest value at 48 h. In contrast, for seeds imbibed in 5 mM guazatine, *OsPAO5* expression in the embryos increased until 6 h after which it decreased and seemed to be repressed by 12 h (**Figure 5C**). These results were consistent with those for PAO activity (**Figure 3**), changes in H_2O_2 content in embryos (**Figure 2**), and germination percentage in the rice seed (**Figure 1**), suggesting that *OsPAO5* is responsible for the PAO level and activity, and has an important role during germination of rice seeds.

DISCUSSION

Involvement of H_2O_2 Generated by PAO Activity during Germination of Rice Seeds

For seeds of dicots, e.g., *Arabidopsis*, lettuce, and tomato, the endosperm cap is the main barrier to germination (Nonogaki et al., 2000; Müller et al., 2006; Zhang et al., 2014; Chen et al., 2016). Weakening of the cap and radicle elongation are required for completion of germination. However, for seeds of monocots, e.g., purple false brome, rice, barley, and maize, the

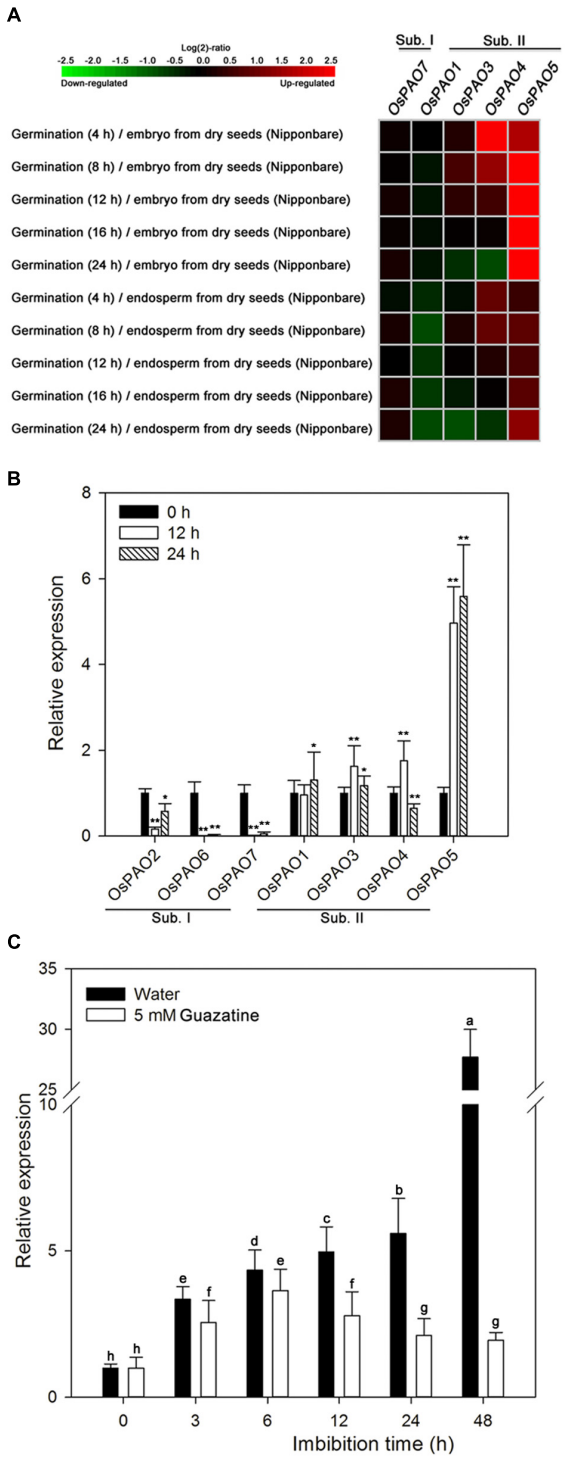

FIGURE 5 | Expression profiles of OsPAO genes during germination of rice seeds. (A) Expression profiles (heat maps) obtained from rice microarray (Os_51k array) data as reported by GENEVESTIGATOR V3. Expression profiles for *OsPAO2* and *OsPAO6* were unavailable. The green/red coding reflects the relative expression levels with dark green representing strong downregulation and dark red representing strong upregulation. The corresponding numerical values are shown in Supplementary Table S2.

(*Continued*)

FIGURE 5 | Continued

(B) The expression levels of *OsPAO1*∼*7* in embryos assayed by qPCR after seeds had been imbibed in water for 0, 12, or 24 h. Data are the mean ± SE of three biological replicates of 30 embryos (∼0.1 g total). Significant differences for the qPCR data were assessed by the Student's *t*-test (**P* < 0.05; ***P* < 0.01). **(C)** *OsPAO5* expression patterns in the embryo assayed by qPCR after seed imbibition of water or 5 mM guazatine at 0, 3, 6, 12, 24, and 48 h. Data are the mean ± SE of three biological replicates of 30 embryos (∼0.1 g total). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Fisher's least significant difference test.

main function of the endosperm is to provide nutritional energy for germination and seedling establishment (Bewley et al., 2013), and the coleorhiza is the main obstacle to radicle protrusion. Emergence of monocot radicles may depend on softening of the coleorhiza, and on the expansive force of the imbibing radicle cells (Gonzalez-Calle et al., 2015). For the study reported herein, we observed that the coleorhiza first protrude from the pericarp and then the radicle and coleoptile emerge from the coleorhiza during germination of rice seeds (**Figure 1**). Thus, the rice coleorhiza functions in a manner similar to that of the endosperm cap in dicot seeds. Endosperm cap weakening and radicle elongation during germination of dicot seeds require cell wall loosening, which involves both enzymes, e.g., mannase and cellulase, and non-enzymatic reactions, e.g., those of ROS (Nonogaki et al., 2010; Zhang et al., 2014; Scheler et al., 2015; Chen et al., 2016). These two types of reactions also appear to be required for germination of monocot seeds. For example, mannase activity has been detected in the coleorhiza and radicle during germination of rice and purple false brome seeds (Ren et al., 2008; Gonzalez-Calle et al., 2015). In our study, we found that the production and accumulation of H_2O_2 was greater in the coleorhiza and radicle than in the coleoptile of germinating rice seeds (**Figure 2**), indicating that H_2O_2 might be involved in the loosening of coleorhiza and radicle cell walls, which is a finding similar to what we found for germination in lettuce seeds (Zhang et al., 2014).

In addition to NADPH oxidases, PAOs and oxalate oxidases are enzymes that produce H_2O_2 (Cona et al., 2006; An et al., 2008). H_2O_2, O_2^-, and $^{\cdot}OH$ have been found to be involved in the loosening of cell walls (Müller et al., 2009). In the apoplast, $^{\cdot}OH$, produced from O_2^- and H_2O_2, may directly cleave wall polysaccharides to help destroy the integrity of the cell (Schweikert et al., 2000; Liszkay et al., 2004) and facilitate completion of germination (Zhang et al., 2014). We found that exogenous H_2O_2 promoted germination of rice seeds, whereas this process was inhibited by the H_2O_2 scavenger, DMTU (**Figure 1**; Ben Rejeb et al., 2015), suggesting that H_2O_2 is necessary for the germination process, a conclusion similar to that found for germination of sunflower seeds (Oracz et al., 2009). When the rice seeds were imbibed in water, the H_2O_2 content and PAO activity increased and paralleled that of the germination percentage. Conversely, for seeds imbibed in the PAO inhibitor, guazatine (**Figures 2** and **3**; Atanasov et al., 2016), germination was strongly inhibited, as was the H_2O_2 content and PAO activity in the embryos. These data indicate that PAO-produced H_2O_2 is essential for germination. Similar results by Zhang et al. (2011)

demonstrated that PAO-produced H_2O_2 promotes germination of lettuce seeds. Moreover, much evidence supports the notion that PAO-generated H_2O_2 regulates such physiological processes as closure of fava bean stoma (An et al., 2008), development of soybean lateral roots (Su et al., 2006) and hypersensitive cell death of tobacco (Yoda et al., 2003, 2006). Additionally, the O_2^- concentration and POD activity were increased in rice seeds when germinated in water (**Figure 2**), suggesting that H_2O_2, O_2^-, and $^{\cdot}OH$ are important to seed germination via their loosening of the coleorhiza cell walls of rice seeds (Schweikert et al., 2000; Liszkay et al., 2004; Müller et al., 2009). Interestingly, guazatine hardly inhibited the rate of O_2^- production but intensely suppressed H_2O_2 production (**Figure 2**), which indicates that guazatine is not an effective inhibitor of O_2^- production but is specific for H_2O_2 generation. Guazatine slightly suppressed POD activity (**Figure 2**), which might indirectly reflect the rate of $^{\cdot}OH$ production (Müller et al., 2009). Consequently, we speculate that a decrease in H_2O_2 production may reduce $^{\cdot}OH$ production and thereby inhibit germination of rice seeds.

Functional Diversity of OsPAO Genes and the Possible Role(s) of OsPAO5 in the Germination of Rice Seeds

Gene duplication is often found to have occurred in eukaryotic genomes and thereby has contributed to biological diversity (Van de Peer et al., 2009; Magadum et al., 2013). Fusion of sequences encoding additional domains after gene duplication can lead to new functions associated with the duplicated gene products (Kaessmann, 2010). We identified 11 PAO homologs in the rice genome, which are distributed on chromosomes 1, 2, 4, 8, 9, and 10 (Supplementary Table S1). These proteins were classified into the four known and well-conserved subfamilies, I, IIa, IIb, and III, which have distinct subcellular locations, domain organizations, and functions (**Figure 4**). These observations suggest that a duplication of an ancestral PAO gene might have led to the expansion of the PAO gene family, which is associated with functional divergence. Unlike OsPAO1–7, members of subfamilies I, IIa, and IIb; *OsPAO8–11* encode histone lysine-specific demethylases, which are involved in control of flowering time and seed dormancy. The N-terminal SWIRM domain found in OsPAO8–11 may be the result of gene fusion, which may, therefore, have resulted in the functional diversity of rice PAO family members. This phenomenon is similar to what we found for NAD(H) kinase and NADPH oxidase (Scheler et al., 2015) family members (Li et al., 2014; Chang et al., 2016).

In plants, PAOs have diversified biochemical properties and physiological functions (Cona et al., 2006; Kusano et al., 2008; Moschou et al., 2008b; Alcázar et al., 2010; Angelini et al., 2010; Fincato et al., 2011; Wimalasekera et al., 2011). To begin with, PAOs were demonstrated the key enzyme for regulating cellular PAs levels which are critical for developmental processes, e.g., embryogenesis (Bertoldi et al., 2004; De-la-Pena et al., 2008), germination (Bethke et al., 2004; Liszkay et al., 2004), root growth (Cona et al., 2005), and flowering and senescence (Kakkar and Sawhney, 2002); for tolerance to stresses such as drought (Alcázar et al., 2006), salinity (Groppa and Benavides, 2008), temperature extremes (Groppa and Benavides, 2008), mineral deficiency, and wounding; and for defense against pathogens (Moschou et al., 2008c, 2009). Far from being only a means of regulating cellular polyamine levels, PAO contribute to important physiological processes through their reaction products [i.e., amino aldehydes, 1,3-diaminopropane (DAP) and hydrogen peroxide (H_2O_2)] that is we focus on. For example, PAs, DAP and H_2O_2 were the key signals associated with development, stress tolerance and defense in plants (Alcázar et al., 2010; Hussain et al., 2011; Wimalasekera et al., 2011). H_2O_2 derived from apoplastic catabolism of PAs acts synergistically with NO for the expression of defense and detoxification genes, and during hypersensitive reaction and developmental programmed cell death (Mittler et al., 2004; Moschou et al., 2008a; Wimalasekera et al., 2011).

Furthermore, PAO homologures includes PAOs, e.g., OsPAO1–7 and AtPAO1–5, and histone lysine-specific demethylases, e.g., OsPAO8–11 (OsHDMA701∼704) and AtPAO6∼9 (AtLDL1–3, AtFLD; **Figure 4**) with the first group catalyzing the terminal catabolism or back-conversion of PAs (Fincato et al., 2011; Kim et al., 2014; Liu et al., 2014a,b) and the second catalyzing the demethylation of histone H3K4 (Shi et al., 2004; Zhou and Ma, 2008; Zhao et al., 2015). These studies suggest that plant PAOs have multiple functions and are involved indirectly in developmental and physiological processes. The rice PAO homologs are divided into multiple subfamilies suggesting the functional diversity of these proteins as well.

Seed germination is a complex physiological and biochemical process that involves a series of signal transduction and regulation of gene expression (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Gomes and Garcia, 2013; He and Yang, 2013; Han and Yang, 2015). We confirmed that PAOs are involved in germination of rice seed and that they regulate H_2O_2 production via oxidative degradation of PAs (**Figures 1–3**). The PAO gene family in rice encodes 11 homologous proteins (**Figure 4**), but which of these protein(s) is important to germination was unclear prior to this report. Thus, we conducted microarray analysis and qPCR (**Figure 5**) to identify which of the seven PAOs (OsPAO1–7) is most important for germination. We found that *OsPAO5* is potentially the most important gene as its expression profile increased during the time seeds were imbibed in water. Transcription was somewhat inhibited by the specific PAO inhibitor guazatine as was accumulation of H_2O_2 and the PAO activity in the imbibed seeds.

Taken together, although major structural differences exist in seeds of monocot and dicot, the underlying mechanisms for regulation of seed germination seem similar as coleorhiza or endosperm weakening, respectively, and radicle elongation are required for all seed germination (Gonzalez-Calle et al., 2015). As with many dicot seeds, including those of lettuce (Zhang et al., 2014) and tomato (Morohashi, 2002), H_2O_2 specifically accumulates in the coleorhiza and radicle of the germinating rice seed. Moreover, PAOs, as enzymes that produce H_2O_2 by oxidation of PAs, are increasingly activity in imbibed rice seeds. The observed changes in germination percentage, H_2O_2 production, and PAO activity in the germinating rice seeds, suggest that H_2O_2 produced by PAOs is important to coleorhiza-limited germination. Given the functional classifications of the

rice PAOs and the transcript expression of their genes during germination, *OsPAO5* probably is the gene that encodes most of the PAO activity that we observed during germination. Future studies should focus on the physiological role(s) of OsPAO5 and other OsPAOs, and the involvement of H_2O_2 and OsPAOs during germination, as they will help develop genetic methods, e.g., gene knockout and over-expression, that will increase our knowledge of how germination occurs.

AUTHOR CONTRIBUTIONS

B-XC and W-YL conceived and designed the experiments, analyzed the data and wrote the paper; B-XC and Y-TG performed all the experimental research and W-YL carried out bioinformatics analysis and provided funding; B-XC, W-YL, and Z-JC critically revised the manuscript; W-NZ offered the help for photography; Q-JL and ZC for the revision of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01219

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B-XC and W-YL conceived and designed the experiments, analyzed the data and wrote the paper; B-XC and Y-TG performed all the experimental research and W-YL carried out bioinformatics analysis and provided funding; B-XC, W-YL, and Z-JC critically revised the manuscript; W-NZ offered the help for photography; Q-JL, ZC for the revision of the manuscript. All authors read and approved the final manuscript.

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Hydrogen Peroxide and Polyamines Act as Double Edged Swords in Plant Abiotic Stress Responses

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The specific genetic changes through which plants adapt to the multitude of environmental stresses are possible because of the molecular regulations in the system. These intricate regulatory mechanisms once unveiled will surely raise interesting questions. Polyamines and hydrogen peroxide have been suggested to be important signaling molecules during biotic and abiotic stresses. Hydrogen peroxide plays a versatile role from orchestrating physiological processes to stress response. It helps to achieve acclimatization and tolerance to stress by coordinating intra-cellular and systemic signaling systems. Polyamines, on the other hand, are low molecular weight polycationic aliphatic amines, which have been implicated in various stress responses. It is quite interesting to note that both hydrogen peroxide and polyamines have a fine line of inter-relation between them since the catabolic pathways of the latter releases hydrogen peroxide. In this review we have tried to illustrate the roles and their multifaceted functions of these two important signaling molecules based on current literature. This review also highlights the fact that over accumulation of hydrogen peroxide and polyamines can be detrimental for plant cells leading to toxicity and pre-mature cell death.

Keywords: polyamines, hydrogen peroxide (H_2O_2), reactive oxygen species (ROS), nitric oxide (NO), abscisic acid (ABA), abiotic stress tolerance

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INTRODUCTION

Life and stress go hand in hand for all living organisms but in case of plants, being sedentary organisms, stress has to be dealt with in a special way. Plants are subjected to constant environment changes forcing them to fine tune their metabolic processes in order to maintain a steady state balance of the energy production and consumption. A dedicated-signaling network influencing the three main metabolic processes—photosynthesis, respiration, and photorespiration—help in overcoming the imbalance, thereby maintaining growth, and productivity. The main fallout of metabolic imbalance is oxidative stress caused due to the excess production of reactive oxygen species (ROS). Therefore, in order to maintain normal growth and development the plants orchestrate a myriad of stress responsive metabolites like proline and polyamines, along with several antioxidative enzymes, that help to detoxify the ROS. Recent studies have also revealed the capability of ROS to act as signaling molecules in activating defense responses (reviewed by Gill and Tuteja, 2010; Gupta et al., 2013a; Pál et al., 2015; Saha et al., 2015). Thus, ROS are considered nowadays as not only toxic byproducts of aerobic metabolism with strictly controlled cellular levels, but they also function as signaling agents regulating many biological processes and producing pleiotropic effects (Gadjev et al., 2008; Mittler et al., 2011).

Polyamines—putrescine, spermidine, and spermine—which are present in multitude of living organisms are a group of low molecular weight polycations with diverse physiological and developmental functions essential for events such as senescence and stress responses (Roy et al., 2005; Gupta et al., 2014; Nahar et al., 2016). Counterbalancing cellular levels of ROS, in order to maintain a healthy environment for the cells to thrive, is one of the major roles played by polyamines (Miller et al., 2010; Saha et al., 2015). Hydrogen peroxide (H_2O_2) is one of the key ROS molecules produced in living cells from various internal sources. Particularly in plants, the major processes that lead to the production of H_2O_2 involve photorespiration or C_2 cycle, which includes three different organelles—chloroplast, mitochondria, and peroxisome. Of these, mitochondrial and chloroplastidial electron transport chain and oxidation of fatty acids in the mitochondrial matrix play a major role in contributing to the H_2O_2 pool within the cell. In addition, pathogenic infections might also induce an oxidative stress leading to oxidative burst. In fact both the production and scavenging of H_2O_2 act in synchrony to tide out plants during stress conditions (Miller et al., 2010).

The paradox of H_2O_2 physiology is indeed an interesting one—on one hand at lower concentrations it initiates various intra cellular signaling activities while at higher concentrations it is malevolent for the cellular metabolites (Gechev and Hille, 2005; Bhattacharjee, 2012). ROS levels when too high might lead to metabolic dysfunctioning of plant cells and at the same time induce nucleic acid, protein, and lipid damages (Anjum et al., 2015). To combat oxidative stress, plants produce metabolites and molecules like polyamines and H_2O_2 (Mittler et al., 2011). Sub-cellular organelles like the mitochondria and chloroplasts are also key regulators in the sense that alterations in carbon metabolism during stress in these compartments also help in metabolic coordination to avoid excessive generation of ROS and oxidative damage (Takahashi and Murata, 2008; Baxter et al., 2014).

The focus of this review is to decipher the roles of the two important players—H_2O_2 and polyamine—either antagonistic or agonistic or both and to try to elucidate a relationship between them, which eventually modulate the signaling cascades that are initiated in response to abiotic stress.

ROS AND POLYAMINES—KEY PLAYERS IN ABIOTIC STRESS RESPONSE

In this modern era of food security, the ever-increasing level of population demands a robust scientific approach for proper harvest and increase of food crops. From drought to salt and metal toxicity to temperature—stress conditions are omnipresent and have to be dealt with properly without creating any adverse effect on the essential metabolome. The crop productivity of the entire world in this era of food security remains a matter of great concern. It has been observed for a long time that most damage to crop plants in fields occur when two or more stresses are prevailing (Mittler, 2006). Most recent studies indicate that the plant's responses to two or more factors are unique and differ from the response to one factor only. For example, subjecting the plants to only drought stress leads to high content of proline, but subjecting the same species to drought combined with high temperature leads to high content of sucrose and other sugars, but not proline. Hence, Mittler (2006), studying all prevailing abiotic factor has suggested treating this situation as a new stress condition that he called "stress combination." Several studies have established the role of ROS as a key signaling molecule in initiating defense mechanism in response to environmental stresses and pathogen infections by modulating pathways involved in different developmental processes and programmed cell death (PCD) (Mittler et al., 2011; Baxter et al., 2014). Different abiotic stress factors such as drought and salinity have been found to augment the production of ROS and lead to ROS-associated injury (Miao et al., 2006; Abbasi et al., 2007; Zhu et al., 2007; Giraud et al., 2008). ROS scavenging properties have also been identified by the increased cellular accumulation of sugar alcohols such as mannitol, sorbitol, and inositol. Transgenic tobacco with increased mannitol concentration confers protection to the cell from oxidative damages by increasing its scavenging capacity. Moreover, mannitol accumulations do not show any deleterious effect on plants, thus proving that sugar does not show any malicious feedback (Bolouri-Moghaddam et al., 2010). Intensity of light has also remained a potent stress for plants and the triggering of common pathways has been illustrated in a number of studies. The main effect of high light intensity is the damage to the chloroplasts and the antennae complex (Davletova et al., 2005; Moller et al., 2007; Triantaphylidès et al., 2008). The maximal efficiency of PSII and the accumulated ROS, share a linear relationship between each other. Thermal damage to photosystems leads to less absorption of photon. In severe thermal stress conditions, the photons absorbed by PSI and PSII are considered as surplus electrons, and serve as the source of ROS (Moller et al., 2007). High atmospheric CO_2 levels have been found to stimulate photosynthesis in C3 plants because of decreased photorespiration, which is widely accepted as a major source of H_2O_2 (Miller et al., 2008). Therefore, low CO_2 level activates ROS generation leading to the activation of abiotic and biotic stress responses (Munné-Bosch et al., 2013). H_2O_2 mediates the expression of a number of genes—H_2O_2 induced transcripts have been found to play an important role and encoded proteins with functions such as metabolism, cellular energy production, protein destination and transport, cellular organization and biogenesis, cell rescue of defense, and transcription (Desikan et al., 2001; Neill et al., 2002) (**Figure 1**). Activation of signaling molecules such as Ca^{2+}, salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), ethylene, nitric oxide (NO) have been reported to be directly or indirectly linked with H_2O_2 signaling and vice versa. More studies have provided evidence that H_2O_2 itself is a key signal molecule mediating a series of molecular stress responses, being a part of the signaling cascade (Desikan et al., 2004), and activating many other important signal molecules (Ca^{2+}, SA, ABA, JA, ethylene, NO) of plants (Schieber and Chandel, 2014; Vaahtera et al., 2014). These signal molecules function together

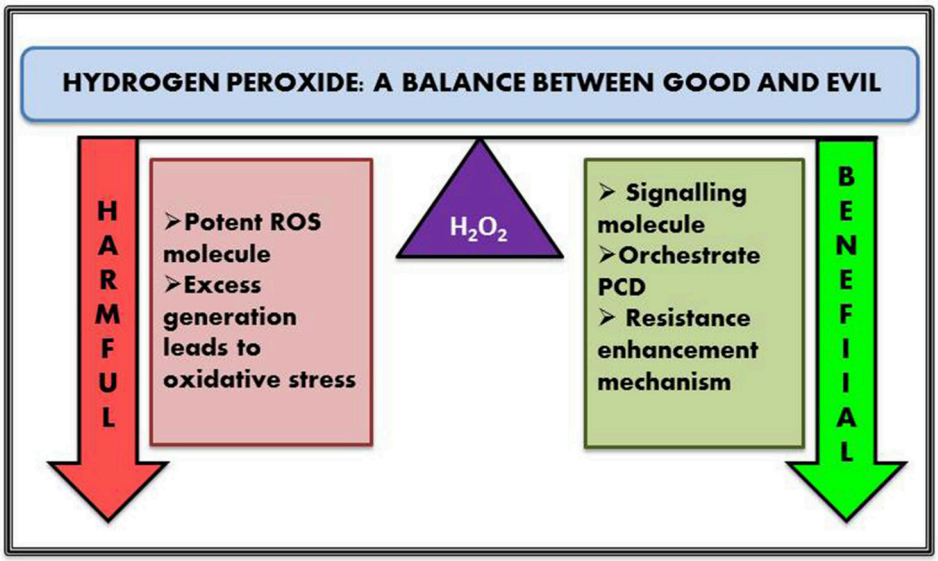

FIGURE 1 | Dual role of intercellular hydrogen peroxides.

and play a complex role in signal transduction of resistance responses, and growth and development in plant. H_2O_2 as a signaling molecule has drawn attention in the last decade or so and ample evidence has been found which supports these assumptions (Dat et al., 2000; Baxter et al., 2014; Saha et al., 2015). Apart from its role as master player in plant stress response regulator it was also reported as the most basic key ingredient in regulating several physical and physiological aspects of plant growth and development such as cell cycle, stomatal movement, photosynthesis, photorespiration, and senescence (Bright et al., 2006; Mittler et al., 2011).

Exogenous application of molecules like polyamines has remained an important genre of studying ways to ameliorate stress in plants (Roy et al., 2005; Farooq et al., 2009; Gupta K. et al., 2012; Gupta S. et al., 2012; Sengupta et al., 2016). Abiotic stress causes drastic changes in the pathways involved in the metabolism of N_2 and polyamine. The exact role of these polycationic molecules had remained undefined for many years. With the use of model systems like *Arabidopsis thaliana*, there has been a global approach in deciphering the role of the polyamines and unveiling its metabolic pathway (Ferrando et al., 2004). According to recent studies, the maintenance of proper equilibrium of its catabolic and anabolic pathways along with the modulations of H_2O_2 level during these processes indeed help plants to tide over stress and adapt properly to the surrounding environment. Recent studies indicate that the redox gradient across the plasma membrane is a key sensor of global climatic change and a crucial regulator of redox signaling (Munné-Bosch et al., 2013). The impact of this global climate change on agriculture will be enormous and it is essential for our survival to note various aspects of H_2O_2 function and crosslinks with regulatory molecules like polyamines.

POLYAMINES—ANABOLISM, CATABOLISM, AND CONJUGATION

Polyamine biosynthesis in plants progress through the decarboxylation step(s) of ornithine or arginine (**Figure 2**). In the presence of enzymes, namely either ornithine or arginine decarboxylases (ODC or ADC), the diamine putrescine is formed. The ADC pathway, which yields putrescine, consists of three sequential enzymatic steps, beginning from agmatine iminohydrolase (AIH) and ending at N-carbamoyl putrescine amidohydrolase (CPA). Sequential addition of aminopropyl groups to putrescine and spermidine leads to synthesis of higher molecular weight polyamines by the activity of spermidine synthase and spermine synthase. SAM decarboxylase helps in generating the amino-propyl groups (**Figure 2**). Analysis and characterization of genes encoding these enzymes in *Arabidopsis* has shown that in this plant there is only ADC activity and the ODC activity is not detectable (Hanfrey et al., 2001), whereby indicating that putrescine is produced exclusively through the ADC pathway. Moreover, it has been found that in *Arabidopsis* there are two genes encoding ADC (*ADC1* and *ADC2*), a single gene, each for AIH and CPA (Janowitz et al., 2003; Piotrowski et al., 2003) and at least four for SAM decarboxylase (*SAMDC1, SAMDC2, SAMDC3,* and *SAMDC4*) (Urano et al., 2004). Also, it has been further observed that there are two genes for spermidine synthase (*SPERMIDINES1* and *SPERMIDINES2*).

Both anabolic and catabolic pathways regulate the levels of polyamine whose intracellular levels depend not only on their biosynthesis but also on catabolic and conjugation reactions. Two main enzymes namely amine oxidases, in particular diamine oxidases (DAO) and polyamine oxidases (PAO), catalyze the deamination of polyamines. DAOs display high affinity for diamines, while PAOs oxidize secondary amine groups

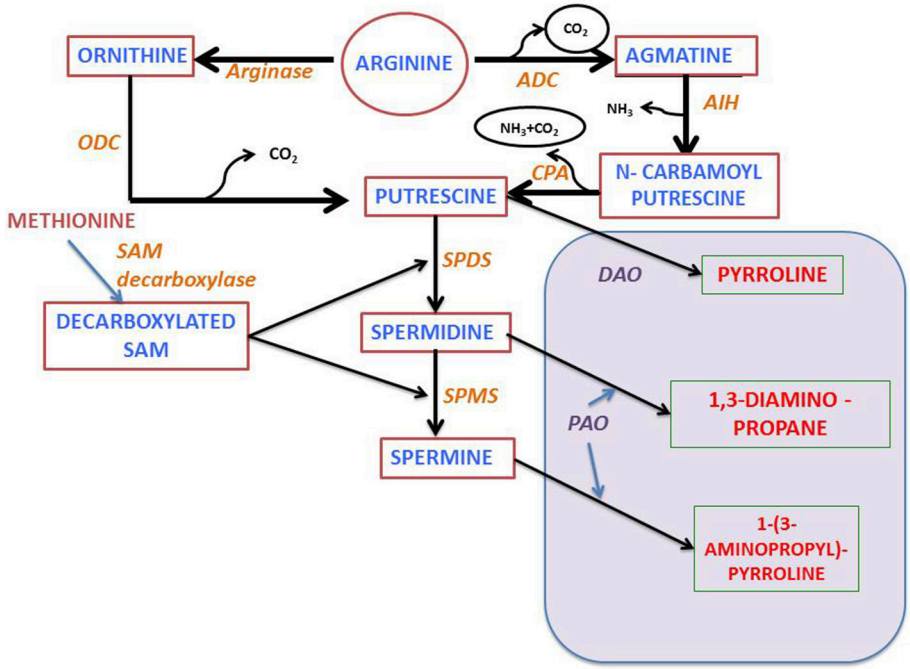

FIGURE 2 | Polyamine metabolism: PA, Polyamine; ODC and ADC, Ornithine or arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, N-carbamoylputrescine amidohydrolase; DAO, diamine oxidase; SPDS, spermidine synthase; SPMS, spermine synthase; SAM, S-adenosylmethionine; PAO, polyamine oxidase (see text for further details).

from spermidine and spermine. The *A. thaliana* genome contains 12 genes with sequence homology to *DAO*s from other species, and 5 *PAO* genes (Moschou et al., 2012). Polyamines not only form base conjugates but also conjugate with other macromolecules like proteins and hydrocinnamic acid (reviewed by Hussain et al., 2011; Gupta et al., 2013a). Enzymes which are involved in the conjugation reactions are transglutaminase and putrescine hydroxycinnamoyl transferase respectively (Bagni and Tassoni, 2001). In *Arabidopsis*, one gene encoding the enzyme transglutaminase has been characterized (*AtPng1*) (Della Mea et al., 2004). However, till date putrescine hydroxycinnamoyl transferase encoding gene has not yet been identified. Involvement of secondary metabolites in plant abiotic stress response has been widely studied using *A. thaliana* as a model plant (Ferrando et al., 2004). Arabidopsis whole genome sequencing (Arabidopsis Genome Initiative 2000) enabled researchers to identify all the genes that are involved in polyamine anabolic and catabolic pathways. Genome-wide expression profiling of genes involved in abiotic stress responses provided a global view on plant defense mechanism in light of polyamine metabolism (e.g., using AtGenExpress; Kilian et al., 2007). Apart from genome level analysis, transcriptomic approach is another well-established method used for recognizing the underlying interrelationship among the abiotic stress induced transcripts along with their *cis*-regulatory elements (Kilian et al., 2007). A similar kind of work was carried out by Ma and Bohnert (2007), where they correlated the presence of specific *cis*-regulatory motifs with the expression profile of several stress induced genes. They observed that these stress regulatory motifs are profoundly involved in modulating the functions and expression of genes that show differential responses when exposed to abiotic stress. Gong et al. (2016) suggest that SlSAMS1 (S-adenosylmethionine synthetase 1) function as a stress mediatory element enhancing alkali stress tolerance through polyamine and H_2O_2 cross-linked networks in tomato callus. This provide us with a new insight in plant's alkali stress tolerance mechanism.

Several genes involved in polyamine biosynthetic pathways have so far been already reported to be upregulated when exposed to one or combination of one or more abiotic stresses. Putrescine is the first polyamine that is accumulated in cells on exposure to abiotic stress. Interestingly, increase in putrescine concentration leads to the induction of enzymes that are responsible for the conversion of putrescine to spermidine and spermine, through auto-regulation process. Concentration of putrescine, spermidine, and spermine varies greatly within the cell mostly because of the simultaneous degradation pathway which occurs within the cell, whereby generating H_2O_2. This H_2O_2, generated as a result of polyamine catabolic pathway, cause oxidative stress on one hand while on the other it plays an essential role in lignification of cell wall, thus protecting plant from adverse effect of stress. Modulating the level of endogenous polyamine by regulating biosynthetic genes is an important procedure for studying the role of polyamine metabolism in stress alleviation (Alcazar et al., 2006).

H_2O_2 PRODUCTION AND CELLULAR DISTRIBUTION—A NECESSARY EVIL

Generation of ROS is considered as an indispensible outcome of aerobic metabolism, which comes along with its share of goodness and evilness. A plethora of ROS species have been identified in plants including H_2O_2, superoxide anion (O_2^-), hydroxyl radicals (OH), singlet oxygen ($^1O_2$), and nitric oxide (NO) and surprisingly most of them are interconvertible. For instance superoxide molecules on reduction yields H_2O_2, which on further reduction liberates water and hydroxyl radical. Cellular oxidation reactions involving these molecules have just the reverse sequence. Studies have shown that only 0.1% of the total oxygen consumed by the plants is diverted for the production of ROS (Bhattacharjee, 2005). ROS is considered as a necessary evil as it functions in several developmental and adaptive responses in both animal and plant cells while its excess generation leads to severe oxidative damage. So it is necessary to maintain a balance between the beneficial and deleterious effects demonstrated by ROS for proper cellular function. Among the different intracellular ROS species, H_2O_2 is considered as one of the most prevalent one. In contrast to other ROS mentioned above, it has a relatively long half-life and can be produced in all cell compartments. Moreover, as it is highly diffusible, it can easily pass membranes. The endogenous H_2O_2 content of plant cells is usually much higher than that found in animals and bacteria; plant cells happily survive with H_2O_2 levels that would kill animal cells. This tolerance is linked to the presence in plant cells of highly efficient antioxidant systems described in detail later on Costa et al. (2010). It is generated by a number of ways in plants. Malfunctioning chloroplast and mitochondrial electron transport chain serves as one of the major source of H_2O_2 generation in plant cells. The process is carried out by membrane bound NADPH Oxidases, also known as respiratory burst oxidase homologs (rbohs), which are regulated by a unique class of Rho-like proteins called ROPs (Rho-related GTPases from plants) as well as by cell wall-associated peroxidases (Agrawal et al., 2003). NADPH Oxidases initially reduce molecular O_2 to superoxide molecule with simultaneous oxidation of NADH to FAD. Superoxide molecule thus produced is converted into H_2O_2 by the action of another enzyme known as superoxide dismutase. Some form of peroxidases (type III POX), in addition to their role in oxidation of phenolics required for cell wall loosening and stiffening, can generate H_2O_2 coupled with the oxidation of NADH (Andronis et al., 2014). In addition, there are several flavin containing limited-substrate oxidases like peroxisomal glycolate oxidase, glyoxisomal xanthine oxidase and urate oxidase, which directly produce H_2O_2 (Delrio et al., 1992). A sulfite oxidase localized in the peroxisome has also been identified to have a role in production of H_2O_2 (Hänsch et al., 2006). Apart from these, oxidases such as DAO and PAO, which are involved in the polyamine degradation pathways, also serve as source to the H_2O_2 pool. Not only stress but also normal physiological conditions can lead to ROS production as part of various metabolic pathways (Ahmad, 2014). For example, oxygen molecule, which is produced as the byproduct of mitochondrial electron transport chain, is sometimes reduced to superoxides that are in turn dismutated to form H_2O_2. Mitochondrial electron transport chain comprises of four distinct enzyme complexes. They are NADH dehydrogenase (Complex I), succinate dehydrogenase (Complex II), ubiquinone-cytochrome C oxidoreductase (Complex III), and cytochrome oxidase (Complex IV). Electron transfer occurs involving either complex I, III, and IV or complex II, III, and IV, leading to the generation of molecular O_2. The molecular O_2 generated can be reduced to superoxide and the reduction is catalyzed by ubiquinone of complex III, which serves as one of the major site for ROS—such as superoxide and H_2O_2 generation. Complex III bears two ubiquinone binding sites, one is located near the inner surface of the inner mitochondrial membrane while the other one is on the outer surface, which indicates the presence of ROS on both luminal and matrix side of this membrane. Not only the mitochondrial electron transport chain, but the chloroplast electron transport chain also transfers electron from photosystem II to NADP thereby yielding reduced NADPH which is used during the Calvin cycle for reduction of CO_2. This also serves as a potent site for superoxide anion and H_2O_2 generation. Other subcellular organelles that actively participate in H_2O_2 production are peroxisomes and glyoxisomes (present only in plants) which carries out several reactions including beta oxidation of fatty acids and light dependent oxidation of glycolate to glyoxylate by glycolate oxidase (Foyer and Noctor, 2005) (**Figure 3**). Thus, it is understandable that generation of H_2O_2 is an irrevocable process irrespective of its consequences.

H_2O_2 plays a versatile role in plants—at mild concentration it acts as a signal molecule and is involved in the alleviation of various abiotic and biotic stresses (Jaspers and Kangasjarvi, 2010; Mittler et al., 2011; Dietz et al., 2016 and its references). At the same time higher cellular concentration of H_2O_2 orchestrates unwarranted PCD and leaf senescence in plants (Dat et al., 2000; Gadjev et al., 2008). H_2O_2 also takes part in plant cell wall reinforcement (lignification, cross-linking of cell wall structural proteins), phytoalexin production and resistance enhancement against different forms of stresses (Gill and Tuteja, 2010; Ahmad, 2014). In case of biotic stress, H_2O_2 production in plants might trigger killing of the pathogen either directly or by prompting defense genes to limit infection by the microbe. Hypersensitive responses are the master players behind establishment of plant immunity against disease causing pathogens. It is another well-known approach that leads to PCD in plant thus inhibiting pathogen invasion. H_2O_2 is the key signaling molecule that actively participates in mediating hypersensitive responses by triggering localized host cell death. It has been also reported to play a crucial role in regulating hormone mediated signaling and vice versa (Pei et al., 2000). A classic example of hormone-mediated response is stomatal closure, where presence of H_2O_2 is perceived by histidine kinase receptor ETR1, which further transduces the signal and ensures the closing of stomata (Bright et al., 2006). Efficiency of any signaling molecule lies in the fact that they are rapidly produced and removed immediately once it has accomplished its role, and H_2O_2 fits the bill perfectly. They are produced rapidly by various cellular processes and quenched promptly. In addition,

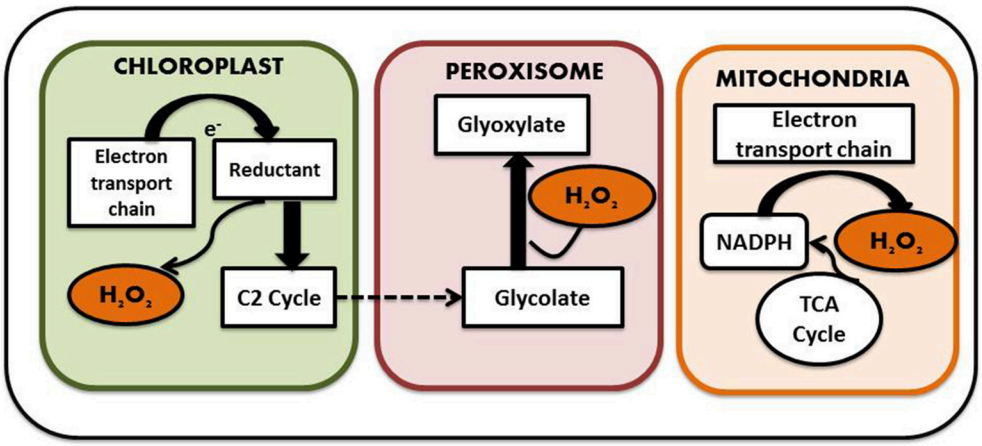

FIGURE 3 | Subcellular localization of H_2O_2 (see text for details).

it can easily react with different biomolecules such as membrane lipid, carbohydrate, protein and DNA and can effortlessly diffuse through aquaporins, thus generating further molecular responses. Apart from its role in hypersensitive reaction and PCD, H_2O_2 induces the expression of glutathione-S-transferase and glutathione peroxidase encoding genes. H_2O_2 treated *E. coli* cells exhibited 140 upregulated mRNA transcripts in DNA microarray experiments—thus revealing the role of H_2O_2 in signal transduction (Li et al., 2001). A similar kind of study was carried out by Desikan et al. (2001) in arabidopsis where about 175 non-redundant EST's were reported those of which are modulated by H_2O_2. Generally signaling molecule activates its receptor by complimentary binding—however, in case of H_2O_2, signal transduction occurs via chemical reactions. Oxidation of cysteine residue of a receptor protein is considered as one of the major path for mediating intracellular signals (Paulsen and Carroll, 2010). There are also evidences that suggest their interaction with secondary messengers such as MAP kinase and their activation. These activated MAP kinase molecules in turn activate different transcription factors thus initiating intricate signaling cascades (Asai et al., 2000). It is interesting to note that H_2O_2 generated from different cellular organelles unveil a plethora of molecular responses. For example H_2O_2 produced from chloroplast was observed to be involved in early signaling responses such as activation of transcription factors and secondary messenger biosynthetic genes while H_2O_2 generated from peroxisomes are mostly involved in upregulating the genes involved in protein repair pathway (Sewelam et al., 2014).

H_2O_2 seems to share a unique inter-relationship with NO and Ca^{2+}. H_2O_2 and NO together have been reported to play an essential role in plant developmental and physiological processes such as growth of pollen tube, growth and development of root, closing of stomata, delayed senescence etc. (Serrano et al., 2012; Huang et al., 2015; Iakimova and Woltering, 2015; Shi et al., 2015). Not only developmental processes, they together play an active role in abiotic stress alleviation as well. They protect drought stressed leaf mesophyll tissue from damage and also increase the activity of myo-inositol phosphate synthase in drought stressed plants (Liao et al., 2010). They have been reported to increase low temperature tolerance by mediating polyamine oxidation in *Medicago* (Guo et al., 2014). There are several other evidences that confirm the role of both H_2O_2 and NO in remitting abiotic stress. However, the mechanism behind their interaction is still not very clear. Most probably H_2O_2 functions as a cofactor in endogenous NO synthesis. This view has been endorsed by the findings of Lin et al. (2012) and Shi et al. (2015). NO on the other hand can regulate stomatal closure in H_2O_2 mutant and in wild type plant treated with H_2O_2 scavengers and inhibitors. Thus, it can be clearly said that both of their production pathways are inter-related and can regulate production of each other. Other than with NO, H_2O_2 has been found to share a distinctive bond with Ca^{+2} as well. Endogenous Ca^{2+} influx increase in arabidopsis root epidermis on application of exogenous H_2O_2. This increase in Ca^{2+} influxes in the root plasma membrane of arabidopsis seedling leads to root elongation. Ca^{2+} influx is probably mediated by H_2O_2 dependent activation of Anexin 1 protein, which is a Ca^{2+} transport protein, thus promoting its growth and development (Demidchik et al., 2007; Richards et al., 2014; Han et al., 2015). Another striking example that demonstrates the inter-relationship between H_2O_2 and Ca^{2+} is H_2O_2 dependent adventitious root formation, which involves Ca^{2+} signaling. In fact the removal of intracellular Ca^{2+} prevents formation of adventitious root. Pei et al. (2000) established a clear association between H_2O_2, Ca^{2+} and stomatal closure using patch clamp techniques, thus connecting ABA signaling cascades with stomatal closure mediated by H_2O_2 and Ca^{2+} channels. Thus, all available evidences point toward an intricate crosstalk between H_2O_2 and Ca^{2+}.

So considering all the roles played by H_2O_2—either good or bad—be it as stress alleviator, secondary messenger, chemo-selective signaling molecule or stress inducer, it surely deserves the tag "Jack of many trades."

SCAVENGERS EMPLOYED TO LIMIT THE OXIDATIVE DAMAGE

Generation of ROS and aerobic life goes hand in hand, and these two phenomena are inseparable. So to deal with the situation—living organisms have evolved several ROS scavenging mechanisms—such as administration of enzymatic and non-enzymatic antioxidants that confer protection against oxidative stress. Among the various ROS species that are present within a cell, H_2O_2 is most stable with a half-life of 1 ms while the half-life of others are much shorter, i.e., about 2–4 μs (Bhattacharjee, 2005). In general H_2O_2 is considered as a weak oxidizing agent. A number of investigations have revealed that ROS, especially H_2O_2, plays an important role related to the signal transduction involved in plant adaptation to the changing environment (Pei et al., 2000; Neill et al., 2002; Moon et al., 2003; Terzi et al., 2014; Reczek and Chandel, 2015). Although the presence of H_2O_2 sensor protein still remain unelucidated, it is presumed that H_2O_2 is directly perceived by the redox transcription factors, showing redox sensitivity, which in turn activates the downstream signaling cascades. It also participates in several physiological pathways of plant and activates defense responses to various stresses. H_2O_2 is beginning to be accepted as a second messenger due to some features that are exclusively present in all secondary messenger molecules. It is mobile, small and can diffuse in and out of the cell freely thereby relaying signals between different cellular compartments, thus modulating different biological processes (Neill et al., 2002). However, excess accumulation of H_2O_2 adversely affects the plants by leading to oxidative stress. Therefore, presence of efficient H_2O_2—scavenging mechanisms is a compulsory criterion for all aerobic organisms. Antioxidative enzymes, and certain non-enzymatic antioxidants (Miller et al., 2008; Sung et al., 2011; Saha et al., 2015) work in tandem and maintain a sinuous but delicate balance to detoxify H_2O_2. Among the wide array of antioxidative enzymes that function in scavenging ROS species, Catalase (CAT), Ascorbate peroxidase (APX), and Peroxidases (POX) require special mention as they act directly upon the H_2O_2 molecules, thus reducing it to water. SOD carries out the dismutation reaction by reducing O_2^- molecule to H_2O_2 whereas CAT, APX, GPX are involved in converting H_2O_2 to water thus ensuring its removal. Based on the presence of metal ion, SOD can be classified into three different types—(i) Mn-SOD, which is located in the mitochondrial matrix and peroxisome, (ii) Cu/Zn-SOD, which is present in large quantities in the chloroplast and cytosol, and (iii) Fe-SOD, which is localized only in chloroplastids of higher plants. All of them function together when the plant is exposed to unfavorable conditions thus protecting the cells from probable damage. H_2O_2 which is generated as a result of superoxide dismutation is removed by enzymes such as CAT, five different isoforms of APX and different isozymes of GPX. These biological catalysts are localized in all the organelles where H_2O_2 generating enzymes are present such as peroxisome, glyoxisome, cytosol, mitochondria etc. CAT is the major enzyme involved in the quenching of H_2O_2 with highest enzyme turnover rate. Studies have revealed that about six million H_2O_2 molecules can be converted to H_2O and O_2 per minute by one molecule of CAT, thereby making CAT one of the major enzymes involved in H_2O_2 detoxification. Apart from CAT another enzyme that plays an equally important role is APX, which catalyses the first step in AsA-GSH cycle and works in coordination with ascorbic acid and glutathione (Asada, 2006; Fan and Huang, 2012; Begara-Morales et al., 2013; Jahan and Anis, 2014). Apart from the enzymatic antioxidants some non-enzymatic antioxidants such as α-tocopherol, ascorbic acid (AsA), glutathione etc. also play a vital role in sustaining stable redox state by removing excess ROS from different cellular compartment, thus detoxifying the cell. AsA that is synthesized in mitochondria, is uniformly distributed throughout the plant and serve as a substrate for APX enzyme, which reduces H_2O_2 to water, yielding mono-dehydroascorbate (MDA) in ascorbate-glutathione cycle (AsA-GSH cycle) (Gapper and Dolan, 2006). It helps regulating α-tocopherol level in cells and is also involved in biosynthesis of zeaxanthine pigment which protects the plant from photo-oxidation or photo-bleaching (Foyer and Noctor, 2005; Munné-Bosch, 2007), thus pursuing an important role in oxidative stress tolerance. Several studies have revealed that application of exogenous AsA diminishes the harmful effect of oxidative stress (Hossain et al., 2011). Glutathione, another non-protein thiol, and a non-enzymatic antioxidant, also plays an essential role in H_2O_2 scavenging (comprehensive review by Noctor et al., 2012). Conversion of reduced glutathione (GSH) to oxidized glutathione (GSSG) and its ratio is considered as a marker for determining redox balance within the cell. Functionally it is almost similar to AsA but in addition to that it also plays an eminent role in heavy metal chelating. Therefore, both glutathione and ascorbic acid are now contemplated as the chief ingredients of redox signaling. Moreover, another group of biomolecules that should be mentioned in this context are polyamines. As mentioned earlier, polyamines are positively charged molecules which protect the cell from oxidative damage both directly and indirectly. Directly it functions as an antioxidant itself thus scavenging free radicals and indirectly it has been reported to regulate the level of enzymatic and non-enzymatic antioxidants within the cell (Groppa and Benavides, 2008). Thus, increase in polyamine concentration is often correlated with increase in stress tolerance. However, on the other hand polyamine catabolism is one of the major contributors of H_2O_2 to the H_2O_2 pool. Endogenous polyamines, like H_2O_2, play a dual role by both adding and diminishing the concentration of H_2O_2 within the cell, thereby maintaining a thin line of balance between their molecular concentrations. Since the maintenance of appropriate H_2O_2 concentration within the cell is of utmost importance for carrying out normal physiological and developmental processes and to combat abiotic stress in plants, efficient ROS scavenging mechanism along with adequate polyamine concentration is of vital importance for its sustainable growth and survival.

ROLE OF POLYAMINE IN OXIDATIVE STRESS

Plant stress biologists have marked oxidative stress as an after effect of each and every abiotic stress—be it heavy metal accumulation, excess salinity, high and low temperature or

scarcity of water. Each of these abiotic stresses is associated with the generation of excess ROS, leading to oxidative damage to cellular components. Studies have revealed that application of exogenous polyamines alleviate abiotic stress, thereby conferring stress tolerance. Abiotic stress is known to impair cellular membranes through their interaction with the membrane structure or as a result of ROS-mediated peroxidation of membrane lipids (Anjum et al., 2015). The antioxidative effect of polyamines can be attributed to a combination of their anionic and cationic-binding properties in radical scavenging, inhibiting properties of lipid peroxidation, metal-catalyzed oxidative reaction, and production of H_2O_2 by DAO and PAO (Groppa and Benavides, 2008). Free and bound polyamines are reported to be modulated by UV-B radiation in different plant species (Mapelli et al., 2008) thereby protecting them against ozone damage and ozone-derived oxidative damage (Groppa and Benavides, 2008). H_2O_2 produced by polyamine catabolism may cause activation of antioxidative defense responses. Phenylpropanoid-polyamine conjugates are known to act as antioxidants against ROS and reactive nitrogen species in response to stress conditions (Yamasaki and Cohen, 2006). Shen et al. (2000) reported that spermidine may act as a cellular membrane protectant against chill-induced lipid peroxidation in cucumber through prevention of activation of superoxide-generating NADPH oxidase. As discussed in the earlier section, CAT enzyme plays an essential role in regulating the balance between excess and exact amount of cellular H_2O_2. Moreover, polyamines directly or indirectly modulate the level of CAT enzyme when exposed to abiotic stress (**Figure 4**), thus forming an intricate stress tolerance network. Sung et al. (2011) have demonstrated the role of polyamines in mitigating hypersalinity-induced oxidative stress in a marine green macroalgae (*Ulva fasciata*) by modulation of antioxidative enzyme (FeSOD, MnSOD, CAT, APX) gene expressions. Radhakrishnan and Lee (2013) observed that, in comparison to untreated plants, CAT activity decreases in polyethylene glycol (PEG) treated soya bean plants simulating drought environment. However, the enzyme activity transiently increases when the plants are treated with PEG along with polyamine spermine. A similar kind of experiment was carried out by Farooq et al. (2009), using rice plant as the experimental model. It was observed that exogenous treatment with spermine successfully ameliorated the effect of osmotic stress by accelerating the activity of CAT enzyme. In another study, acid rain fed bean plants showed a sudden burst in the H_2O_2 level which in turn decreased the CAT activity in the cell. This decrease in the CAT activity is probably due to the binding of H_2O_2 molecule to the enzyme active site, thus deactivating the enzyme (Sharma et al., 2012; Mittova et al., 2015). However, with longer exposure to acid rain, activity of CAT was found to increase, hence conferring stress tolerance. However, in bean plants pretreated with polyamines (spermine or spermidine), the rapid generation of H_2O_2 was checked. This may be due to the protective shield imparted by the polyamines on the membrane which stabilize it (Velikova et al., 2000). Verma and Mishra (2005) demonstrated that under conditions of high salt concentration *Brassica* plants show an increased level of H_2O_2 in plants untreated with polyamines, whereas in polyamine treated plants the level of H_2O_2 decreases considerably. It was also observed that CAT activity increases when exposed simultaneously to NaCl and polyamine, rather than when exposed to NaCl alone, thereby establishing the role of polyamines in protecting the plants from oxidative injury (Verma and Mishra, 2005). Exogenous application of polyamines reduced the H_2O_2 and malondialdehyde (MDA) content, and increased the antioxidant levels in drought and cold stressed 15-day-old chickpea plants (Nayyar and Chander, 2004). DNA oxidative degradation by OH^- was inhibited in the presence of spermine in *Mesembryanthemum crystallinum* (Kuznetsov and Shevyakova, 2007) illustrating the efficiency of polyamines as free radical scavengers. According to some research groups, polyamine conjugates show more efficient antioxidant activities compared to their parent compounds (Edreva et al., 2007; Hussain et al., 2011).

Under metal stress CAT shows a differential response. In some plants like *Glycine max, A. thaliana,* and *Capsicum annuum* CAT activity decreases, while in *Brassica juncea, Oryza sativa,* and *Triticum aestivum* its activity increases. Another very interesting observation was reported by Hsu and Kao (2007) where it was shown that pretreating a plant with H_2O_2 increases the CAT activity which in turn protects the plant from cadmium induced oxidative damage. A similar kind of trend was observed in salt treated *Cicer arietinum*. A completely opposite trend was noticed by Sharma and Dubey (2007) in drought treated rice seedling, where CAT activity decreases. This observation was congruent with the observation made by Pan et al. (2003) where CAT activity decreases in *Glycyrrhiza uralensis* seedlings when exposed to both salinity and drought stress. From the above observations it may be concluded that the increase or decrease in CAT activity primarily depends on the nature of sensitivity toward stress of a particular plant. In sensitive variety CAT level tends to increase. On the contrary, in stress tolerant variety the level of CAT activity decreases. Several reports demonstrate that polyamine plays an interesting role in modulating the CAT level thus regulating the H_2O_2 content of the cell. So it is easy to hypothesize an inter-relation between endogenous and exogenous polyamines, CAT enzymes and stress generated H_2O_2. Most probably, they function in a loop. Oxidative stress leads to generation of H_2O_2 which signals activation of CAT enzyme and endogenous polyamine—CAT functions in removal of H_2O_2 molecule and polyamines protect the membrane from oxidative damage thus conferring a protective shield. Application of exogenous polyamines strengthen the ROS removal procedure in varieties where CAT activity decreases in response to stress thus forming a perfect interrelated network of tolerance (**Figure 5**). Polyamines have been instrumental in reducing protein carbonylation and tyrosine nitration while subsequently increasing protein S-nitrosylation.

Previous results have shown that plants employ polyamine catabolism derived H_2O_2 as a defensive contrivance against abiotic stress (Cona et al., 2006). Tanou et al. (2014) have reported increase in intracellular *DAO* and *PAO* activity in plants treated with excess salt (**Figure 6A**). Treatment with NaCl alone have shown to increase both $O2^{\bullet-}$ and H_2O_2 production, indicating existence of an oxidative stress situation. It was inferred that in the presence of salt, endogenous

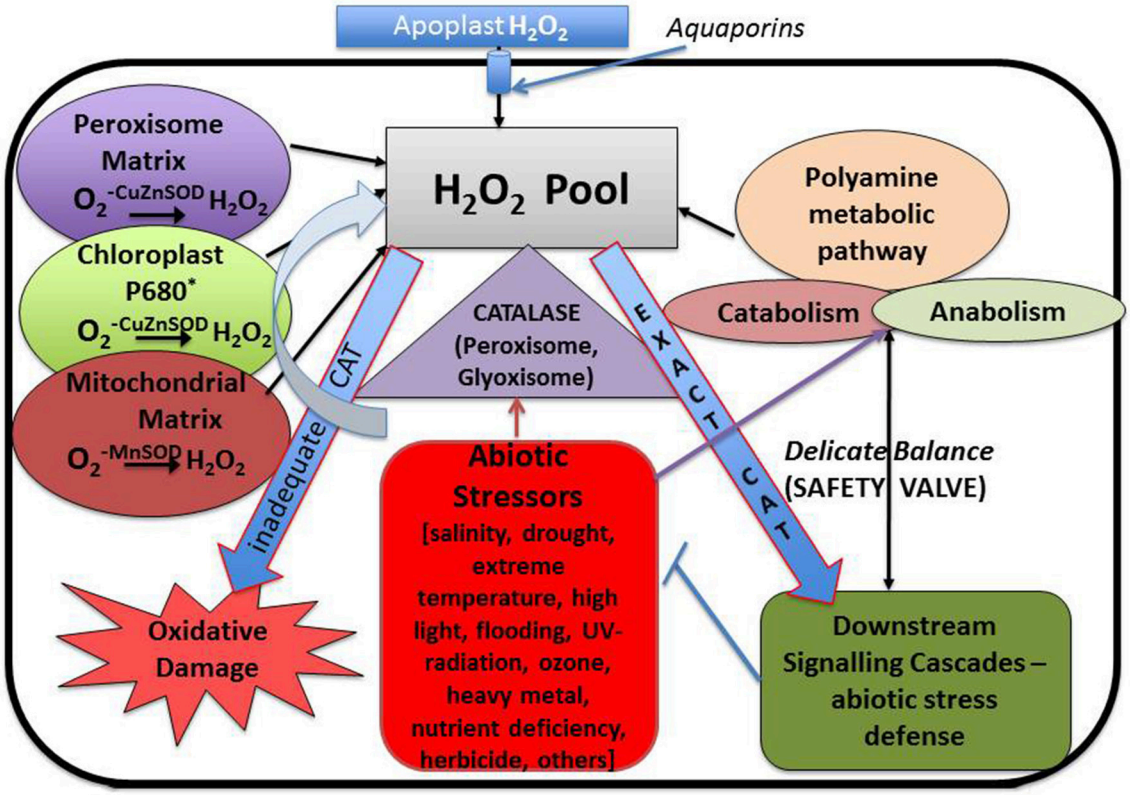

FIGURE 4 | Interrelationship between cellular hydrogen peroxide, polyamine metabolic pathway and different forms of abiotic stresses with special emphasis on the role played by "catalase" antioxidant enzyme.

polyamines induce the generation of $O2^{\bullet -}$. However, exogenous polyamine treatment lowers $O2^{\bullet -}$ level, with significant difference being observed after spermine application. In addition, H_2O_2 content strongly increases in putrescine or spermidine-treated plants compared to those treated with salt alone. Exogenous polyamine application on salt treated plants shows an increase in endogenous polyamine level when compared to plants which are not treated with polyamines, thereby confirming the beneficial role of extracellular polyamine in mitigating salt stress (Shi et al., 2010). Polyamines have been reported to be taking part in inter-organ signals in plants. Moreover, it was observed that putrescine administration evoke spermidine accumulation in roots on exposure to salinity, whereas spermidine treatment enhances spermine production in leaves, illustrating the metabolic conversions of polyamines in the case of the entire plant. Likewise, the addition of spermine increases the endogenous spermidine and putrescine concentration in roots whereas spermidine application increases leaf putrescine concentration in salt treated citrus, thus depicting the possible conversions that might occur on exposure to a single polyamine under abiotic stress conditions (Tavladoraki et al., 2006; Moschou et al., 2008a,b). Studies have divulged that exogenous application of polyamines in salt treated roots stimulates polyamine biosynthetic genes in the leaves, asserting its universal systematic role (Kuznetsov et al., 2002). Further evidences came from the work of Tassoni et al. (2008), who demonstrated the advantageous effect of free spermidine on Arabidopsis flower under high salt concentration. Another extremely interesting phenomenon observed in *Vitis vinifera* revealed that stress exposure causes an immediate rise in the putrescine level; however, increase in spermine/spermidine level occur much later (Liu et al., 2011). Photochemical efficiency of PSII which is often hampered by stress was found to be enhanced by external application of putrescine (Zhang et al., 2009). Rice plants treated with spermidine for the first 7 days after germination followed by their continuous exposure to salinity till maturity shows a better grain yield and increased ion content in comparison to those that are not treated with spermidine for the initial 7 days (Saleethong et al., 2013). Thus, it is evident that ROS scavenging mechanisms in co-ordination with polyamines play important role during plant abiotic stress adaptation.

H_2O_2 AND POLYAMINES—THE DUAL ROLE

The actual cause of cell death induced in plants by oxidative stress still remains a conundrum. What remains a pertinent question is—whether PCD is induced by the ROS or is it the ROS levels itself which causes the cells to decease. Polyamine-derived H_2O_2

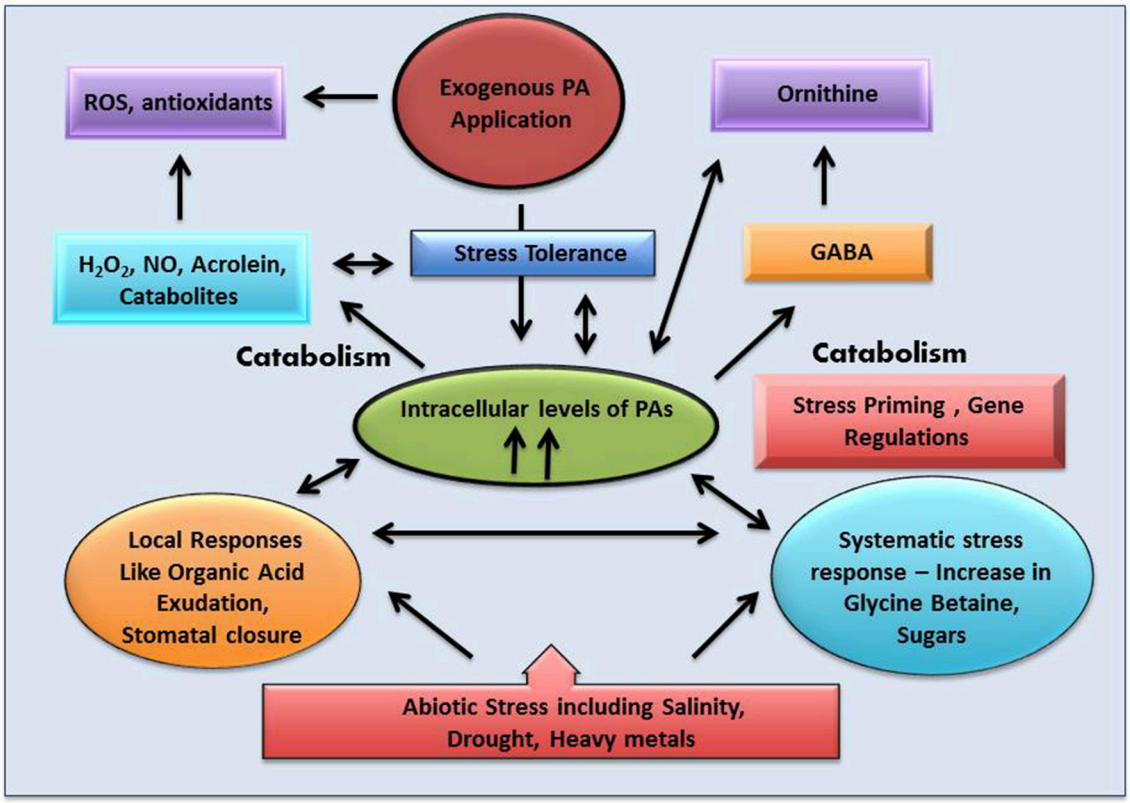

FIGURE 5 | An overview of the role of polyamine (PA) in plant abiotic stress tolerance.

has been shown to participate in stress-induced cell wall stiffening and maturation (Angelini et al., 2010). In HIV-induced neuronal toxicity, Spm oxidase has been shown to act as a mediator of ROS production (Capone et al., 2013). Involvement of H_2O_2 in polyamine-induced cell death has been demonstrated in tobacco (*Nicotiana tabacum*) (Iannone et al., 2013) (**Figure 6A**). It is in this context that the level of H_2O_2 has drawn keen interest by raising doubts in the minds of researchers as to the way in which it leads to cellular damage inducing PCD, and the very fact that it may require a higher level of ROS to exterminate cells by direct oxidation. Studies applying oxidative stress to mutants deficient in different PCD pathways will be able to throw some light on this very question.

Many questions related to ROS metabolism have remained unanswered till date. We are thus trying to address these questions by considering the agency of molecules like H_2O_2 and polyamines both of which work toward a common target of stress tolerance. Intra-cellular levels of polyamines play a pivotal role by regulating a large number of processes through its metabolic pathways, including both anabolism and catabolism. Polyamine catabolism leads to an increase in level of H_2O_2, in turn influencing both stress damage and the response to stress damage (Pottosin and Shabala, 2014). Other by-products of the catabolic pathways include NO, GABA, acroliens, and others. GABA through TCA along with ornithine and other amino acids also acts as a signaling molecule. Studies demonstrate that external polyamine application not only alters polyamine homeostasis and metabolism systemically, but also affects the levels and the activity of antioxidative enzymes. Polyamines have been reported to be involved in the easing of oxidative stress through stimulation of two transcription factors SoxR and EmrR in *E. coli*. SoxR supposedly induces a superoxide response regulon transcription activator and *sodA* genes. These in turn influence the antioxidant machinery of the bacterial cells. Activation of EmrR and GshA together induces glutathione that has an inhibitory effect on H_2O_2 accumulation (Sakamoto et al., 2015) (**Figure 6B**). Scientists around the world look for such overlapping cues in plants, which help them to design their experiments and hypothesis.

A comprehensive analysis including biochemical, physiological, and molecular assays using microarrays and chips, coupled with proteomics will help us in deciphering the exact pathways both polyamines and H_2O_2 work on. Tanou et al. (2012, 2014) reported that a plant's response to salinity involves a crosstalk between polyamine transduction and oxidative/nitrosative signaling. Bruce et al. (2007) in their work have efficiently highlighted the benefits of long-term adaptation to biotic and abiotic stresses in plants and their evolutionary significance. They hypothesized that the plant retaliation toward any particular stress is regulated by a combination of their innate ability to combat stress as well as from previous exposure to a similar kind of stress. It was also opined that treating a plant

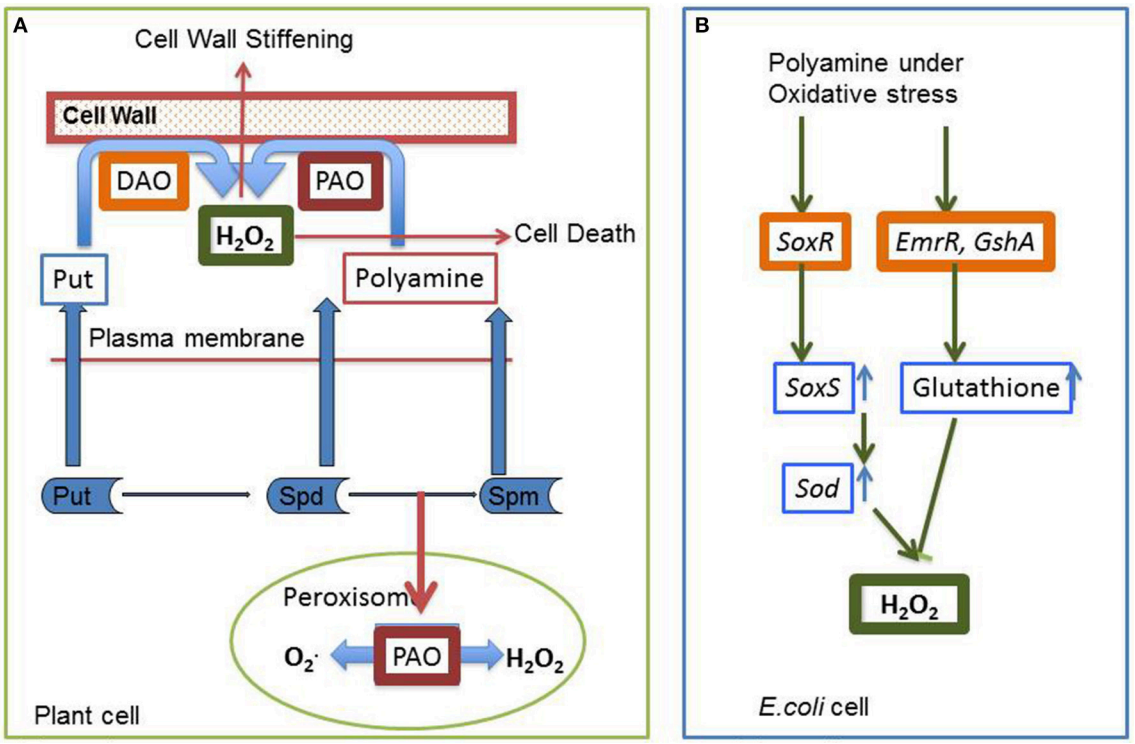

FIGURE 6 | Interaction of polyamine and H_2O_2 (A) In plant cells H_2O_2 is generated during the process of polyamine catabolism, that may be utilized as a signaling molecule, PCD or cell wall stiffening. PAO in peroxisome maintains a delicate balance between $O_2^{\cdot}$ and H_2O_2 **(B)** In simple prokaryotic bacterial cell like in *E. coli*, polyamines have been reported to be involved in ameliorating oxidative stress through stimulation of two transcription factors SoxR and EmrR (see text for details).

with any kind of stress alleviator such as polyamines and in some cases even stress inducers such as H_2O_2 prior to the exposure to stress increases its chances of resistance and perennate (Shi et al., 2010). Li et al. (2015) analyzed the involvement of polyamines in the regulation of H_2O_2 and Ca^{2+} messenger systems associated with antioxidant defense and dehydrins in leaves of white clover resulting in water stress tolerance. However, further investigations are needed for establishing the phenomenon of polyamine-induced stress tolerance associated with H_2O_2 and Ca^{2+} signaling in different plant species and stress conditions.

The exposure to stress during very early development in plants is known as "priming." There are reported examples of the effects of "priming" on physiological and biochemical responses that plants show on recurrent exposure to assortment variety of stresses (Jisha et al., 2013; Jisha and Puthur, 2016). Priming usually begins during seed germination, and might have long-lasting effects on the development of plants. This in fact may lead to adaptation to a diverse type of abiotic and biotic stresses. One can well imagine that it is priming that deals with the epigenetic changes, or markers, which indeed are responsible for carrying information over generations. Modifications of epigenetic regulations due to the changing environment on gene expression are extensively believed to be true; however, the appliance of such epigenetic adaptations is not well understood. The modifications generally occur at the chromatin level, and involve sequence-specific DNA methylation, histone acetylation, sumoylation and other similar abatements. While most of the epigenetic modifications are unwavering within the life of an organism, others are reversible, depending on growth and other regulations, and the rest appear to be transmitted to the subsequent generations through sexual reproduction (Sano, 2010; Shao et al., 2014; Sharma, 2014). From the various functions of polyamines and H_2O_2 studied till date, one can get a fair idea of the major roles played by them in priming for stress. Savvides et al. (2016) have argued about the role of some promising chemical agents such as sodium nitroprusside, H_2O_2, sodium hydrosulfide, melatonin, and polyamines that can potentially confer enhanced tolerance when plants are exposed to multiple abiotic stresses. In the present context it can be debated that increased polyamine accumulation in response to various stress conditions affects the epigenetic modifications of DNA and histones thus conferring stress tolerance. Plant polyamines create cellular responses during abiotic stress through modulation of ROS homeostasis via two distinct mechanisms (Takahashi and Kakehi, 2010). Firstly, polyamines promote ROS degradation by scavenging free radicals and activating antioxidant enzymes during stress conditions (Gupta et al., 2013a,b). Free polyamines are responsible for the detoxification of superoxide anions and H_2O_2, while the conjugated polyamines probably help in the scavenging of other ROS (Langebartels et al., 1991; Berberich et al., 2015). Kuznetsov and Shevyakova (2007) have reported that conjugated polyamines show more antioxidant ability than

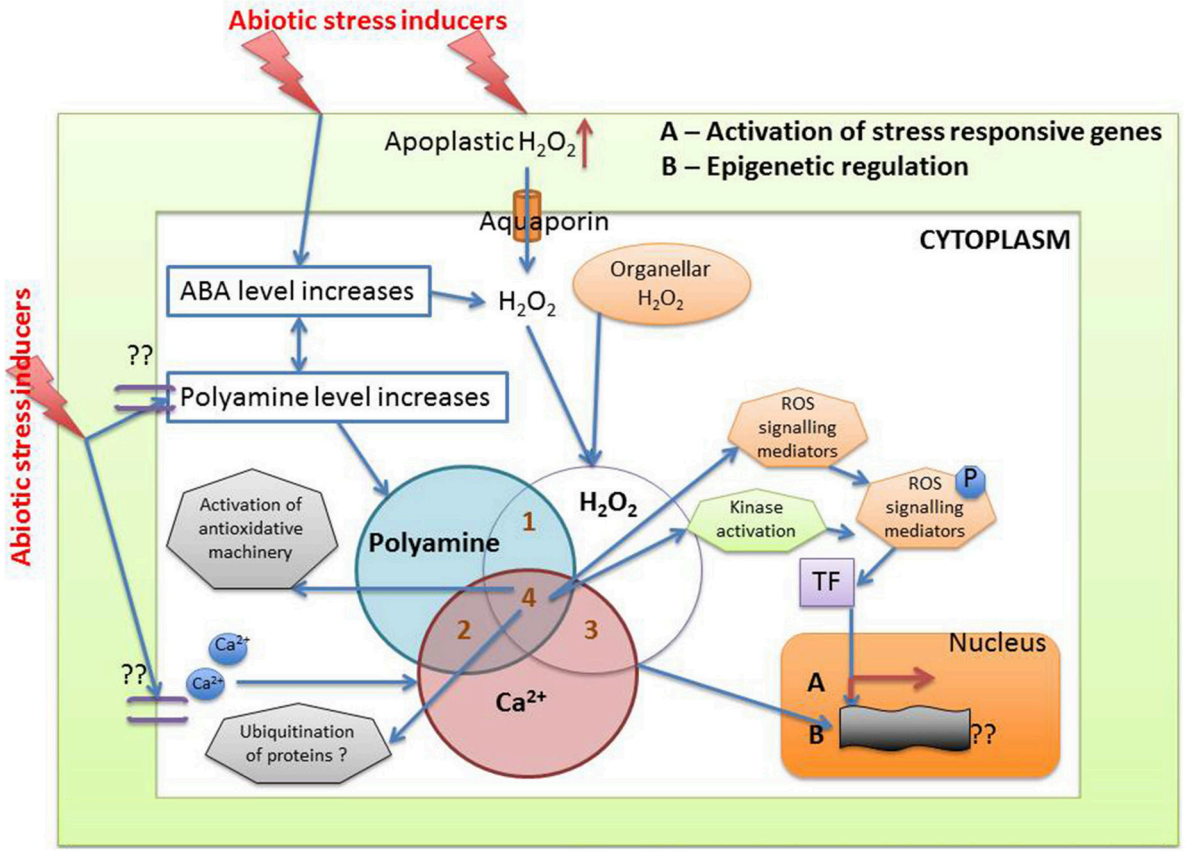

FIGURE 7 | Crosstalk between different metabolites during abiotic stress induced oxidative signaling (see text for details).

free polyamines. Secondly, polyamines promote ROS production through polyamine catabolism in the apoplast (Yoda et al., 2006; Mohapatra et al., 2010; Gupta et al., 2013a,b).

Mellidou et al. (2016) reported that a distinct crosstalk exists between peroxisomal polyamine oxidase and NADPH oxidase for maintaining ROS homeostasis in *A. thaliana* affecting their rate of respiration. They showed that the loss of function in AS-*SAMDC* salt-stressed plants resulted in an enormous increase of H_2O_2 compared to its control genotype. Moreover, higher SOD and elevated level of NADPH-oxidase activity in these SAMDC mutants emphasize the regulation of NADPH-oxidase derived H_2O_2 by SAMDC during salinity-induced stress in *Arabidopsis*. Work done by the same group also showed the NADPH-oxidase dependent stimulation of oxygen consumption in *Arabidopsis*. Peroxisomal AtPAO3 shows a decreased oxygen consumption rate in stark comparison to the loss-of-function in *Atpao3 Arabidopsis* plants, which show increased consumption through the AOX pathway (Andronis et al., 2014). Diphenyleneiodonium iodide (DPI) [but not ascorbate (ASA)] attenuates this increase, suggesting that NADPH-oxidase is upstream of a respiratory increase mediated by AOX. It is interesting to note that overexpressed *AtPAO3* plants show a balanced production of both $O_2^{\bullet-}$ and H_2O_2, while *Atpao3* loss-of-function plants show a high $O_2^{\bullet-}/H_2O_2$ ratio. Their data clearly suggests a well-defined cross-talk between NADPH-oxidase and AtPAO3 for balancing intracellular ROS affecting the cyt-c/AOX pathway. Lambeth and Neish (2014), while reviewing the relation of NOX (animal homolog of NADPH oxidase) and ROS, emphasized the role Nrf2/ARE signaling module in maintaining intracellular redox homeostasis in animals. However, till date no such pathway has been reported in plants. The activation of immune NADPH-oxidase by MAPK induced WRKY transcription factor throws some light on the transcriptional regulation of ROS in plants (Adachi et al., 2015).

From the above discussion the following mechanism can be hypothesized: abiotic stress increases apoplastic and organellar H_2O_2 along with increased synthesis of higher polyamines and second messengers like Ca^{2+}. Increased polyamine adds further to the H_2O_2 pool, which triggers the activation of antioxidative machinery (enzymatic and non-enzymatic) in the plants. ABA levels are also known to increase during stress, and this can be instrumental in triggering ROS mediated signaling pathways via polyamines. Epigenetic regulation of stress response and involvement of protein degradation also cannot be ruled out. While it is difficult to determine which of these mechanisms, during abiotic stress, is the most important one, it can be envisaged that a well-coordinated defense mechanism comprising of polyamines, Ca^{2+}, H_2O_2 take part in response to oxidative stress in plants (**Figure 7**).

TABLE 1 | Double role of H_2O_2 and polyamines in plant abiotic stress.

Metabolite	Role	Plant	Function	References
H_2O_2	Positive	*Arabidopsis thaliana*	Increased expression of Nucleoside diphosphate kinase (NDP) leading to enhanced tolerance to several biotic and abiotic stresses	Moon et al., 2003
		Vicia faba	Intermediate in ABA signaling in guard cells	Pei et al., 2000
		Arabidopsis thaliana	Second messenger in ABA induced stomatal closure	Miao et al., 2006
		Zea mays	Induces salt tolerance by enhancing antioxidant metabolism and reducing lipid peroxidation in both leaves and roots	Azevedo-Neto et al., 2005
		Rice, Arabidopsis, Maize	Tolerance to biotic and abiotic stress by getting involved in various pathways	Reviewed by—Reczek and Chandel, 2015
		Zea mays	Pretreatment alleviates water loss during stress by increasing the level of soluble stress fighters like polyamine, sugars and proline	Terzi et al., 2014
	Negative	*Arabidopsis thaliana*	Indirectly activates WRKY53 transcription factor that leads to leaf senescence	Gadjev et al., 2008
		Arabidopsis thaliana	Influences Oxoglutarate-dependent dioxygenase gene in the cell death process	Gechev et al., 2005
		Ozone-fumigated Arabidopsis leaves	Apoplastic ROS accumulation as a result of activation of NADPH oxidases—leading to PCD	Joo et al., 2005
		Nicotiana sylvestris	Genetic alteration of the mitochondrial electron transport chain desensitizes the plant to stress-induced cell death	Dutilleul et al., 2003
		Arabidopsis thaliana	Enhanced ROS production such as H_2O_2 during drought induced senescence and heat stress	Lee et al., 2012, 2014
		Nicotiana tabacum	PCD is observed in plants deficient in the major catalase isoforms (ascorbate peroxidase and/or catalase)	Rizhsky et al., 2002
Polyamine	Positive	Sour orange plants (*Citrus aurantium*)	Influences oxidative and nitrosative status of plants exposed to salinity stress	Tanou et al., 2014
		Oryza sativa	Recovers salinity stress induced damage of plasma membrane (PM) and PM-bound H^+- ATPase in salt-tolerant and salt sensitive rice cultivars	Roy et al., 2005
		Arabidopsis thaliana and *Nicotiana tabacum*	Transglutaminases catalyse the conjugation of polyamines to photosynthetic complexes and proteins and lead to enhanced photosynthetic activity under abiotic stress conditions	Hamdani et al., 2011; Ioannidis et al., 2012
		Different wheat cultivars	Different polyamines showed a variable increase during cold hardening	Szalai et al., 2009
		Arabidopsis thaliana	*Arabidopsis thaliana* plants overexpressing a *Cucurbita ficifoia* spermidine synthase gene have been demonstrated to become tolerant to multiple stress factors such as low temperature, freezing temperature, drought, salinity, and herbicide Paraquat	Kasukabe et al., 2004
		Mesembrynthemum crystallinum	Spermine inhibited the oxidative degradation of DNA by OH^-	Kuznetsov and Shevyakova, 2007
	Negative	Tobacco plants	Transgenic tobacco plants overexpressing apoplastic PAO are not able to cope with oxidative burst generated by abiotic factors, causing detrimental effects	Moschou et al., 2008b; Moschou and Roubelakis-Angelakis, 2014
			The same plants mentioned above exhibited increased SOD and CAT expression, which do not exert a protective effect, but rather this increased expression represents an attempt to scavenge surplus H_2O_2 produced by continuous polyamine oxidation, which suggest that constitutive polyamine oxidation leads to chronic oxidative stress	Moschou et al., 2008b; Moschou and Roubelakis-Angelakis, 2014
		Nicotiana tabacum	Induction of hypersensitive cell death by H_2O_2 produced through polyamine degradation	Yoda et al., 2003

(Continued)

TABLE 1 | Continued

Metabolite	Role	Plant	Function	References
		Tobacco (*Nicotiana tabacum*) cultured cells	A gene encoding a tobacco *PAO* was isolated and used to construct RNAi transgenic cell lines. The results suggest that PAO is a key element for the oxidative burst, which is essential for induction of PCD, and that MAP kinase is one of the factors that mediate this pathway	Yoda et al., 2006
		Arabidopsis thaliana	Excess amount of exogenous thermospermine or spermine application resulted in an inhibition of leaf expansion, chlorophyll synthesis, and seed germination	Kakehi et al., 2008
		Arabidopsis thaliana	Exogenous thermospermine might also be oxidized, at least in part, by PAO and negatively affect the stem growth	Kakehi et al., 2008
		Oryza sativa	3-aminopropanal generated by polyamine back-conversion is a highly reactive aldehyde and is spontaneously deaminated to give acrolein. It is well known that in mammalian cell cultures, the toxicity of acrolein is higher than that of H_2O_2	Takano et al., 2012; Ono et al., 2012; Yoshida et al., 2009

This review tries to present the dual role of both H_2O_2 and polyamines in a tabular form (**Table 1**). These evidences suggest that it is often "tricky" to establish a direct relationship between increased levels of polyamines/H_2O_2 and abiotic stress tolerance. Pál et al. (2015) have suggested that the statement "the higher polyamine level the better" cannot be generalized and elevated polyamine content might be the cause of stress-induced injury and/or of some protective mechanisms. We would like to suggest that the intracellular level of H_2O_2 and different forms of polyamines act as the "safety valve" of the whole plant system that is delicately balanced depending on the type and duration of the stress, developmental stage of the plant, the genotype it belongs to, and the type of plant tissue affected. Further studies will enable us to get a better understanding of the role of polyamine and H_2O_2 in plants using several modern era technologies. We earnestly hope that the dual role of these two key players playing a silent role in the backdrop will be unveiled in the near future.

CONCLUSION

Environmental stresses are the key reasons behind massive crop loss throughout the world thus ravaging world agricultural economy. So the primary focus of the entire scientific world is to identify and implement strategies to overcome both abiotic and biotic stresses. In this process, they have identified roles of several molecules that are present within any normal living cell under non-stressful environmental conditions, playing the conventional roles that "Nature" designates them to play. But once the usual environment is replaced with a stressed one they start to function as stress alleviators. Two major examples of such molecules are polyamines and H_2O_2. Both of them have some demarcated responsibilities toward the cells they are produced in, but once they are exposed to an unfavorable environment they show a new dimension of their own which could not have been predicted earlier. Initially it was known that both polyamines and H_2O_2 play a significant role in maintaining physiological and biochemical processes in plants. However, their role in signaling and activating each other in response to stress is a new finding. Their correlation will be an intriguing topic for research in the near future. Some aspects of this interrelationship have already been discussed. But that is not enough to discern "whether polyamine and H_2O_2 functions as a dual-edged sword or not?" Despite the complexity plants are able to sort the stress signals and put up a well-organized defense mechanism. We would definitely be interested to know the intricate molecular mechanism that lies behind this unique inter-relationship and that is where the future prospect of this work lies.

AUTHOR CONTRIBUTIONS

KG contributed in writing the manuscript and drawing the figures. AS and MC contributed in initial drafting of the manuscript, developing some of the figures, and have contributed equally. BG designed and developed the entire manuscript and contributed in writing and drawing the figures. KG and BG are

the corresponding authors and also contributed in research fund management.

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REVIEW
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Reactive Oxygen Species (ROS): Beneficial Companions of Plants' Developmental Processes

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Reactive oxygen species (ROS) are generated inevitably in the redox reactions of plants, including respiration and photosynthesis. In earlier studies, ROS were considered as toxic by-products of aerobic pathways of the metabolism. But in recent years, concept about ROS has changed because they also participate in developmental processes of plants by acting as signaling molecules. In plants, ROS regulate many developmental processes such as cell proliferation and differentiation, programmed cell death, seed germination, gravitropism, root hair growth and pollen tube development, senescence, etc. Despite much progress, a comprehensive update of advances in the understanding of the mechanisms evoked by ROS that mediate in cell proliferation and development are fragmentry and the matter of ROS perception and the signaling cascade remains open. Therefore, keeping in view the above facts, an attempt has been made in this article to summarize the recent findings regarding updates made in the regulatory action of ROS at various plant developmental stages, which are still not well-known.

Keywords: reactive oxygen species signaling, plant growth and development, programmed cell death, seed germination, NADPH oxidases

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INTRODUCTION

Life on the earth began under a reducing atmosphere. About ∼2.7 billion years ago, the introduction of O_2-evolving photosynthetic organisms led to an accumulation of O_2 that changed the reducing environment into an oxidized one. Since then, reactive oxygen species (ROS) have been unavoidable companions of aerobic life (Gill and Tuteja, 2010; Bhattacharjee, 2012). Electron transport systems (ETCs) generally produce ROS by virtue of O_2 being a powerful electron acceptor. ROS such as superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are either the product of oxidation–reduction (redox) reactions, or activated derivatives of O_2, continually generated in chloroplasts, mitochondria, peroxisomes, and glyoxysomes (Sharma et al., 2012; Suzuki et al., 2012; Sandalio et al., 2013; Singh et al., 2015a,b), and also in the cytosol, apoplast, nucleus, and, endomembrane systems (Gechev et al., 2006; Ashtamker et al., 2007). These ROS are highly reactive and toxic, causing oxidative damage to macromolecules such as lipids, proteins, and nucleic acids (Karuppanapandian et al., 2011; Kapoor et al., 2015; Prasad et al., 2015). In photosynthetic tissues (leaves), chloroplasts are the major sources of ROS generation in plants (Asada, 2006; Dietz, 2016; Takagi et al., 2016; **Figure 1**; **Table 1**). Photosynthetic electron transfer chains of chloroplast produce high amounts

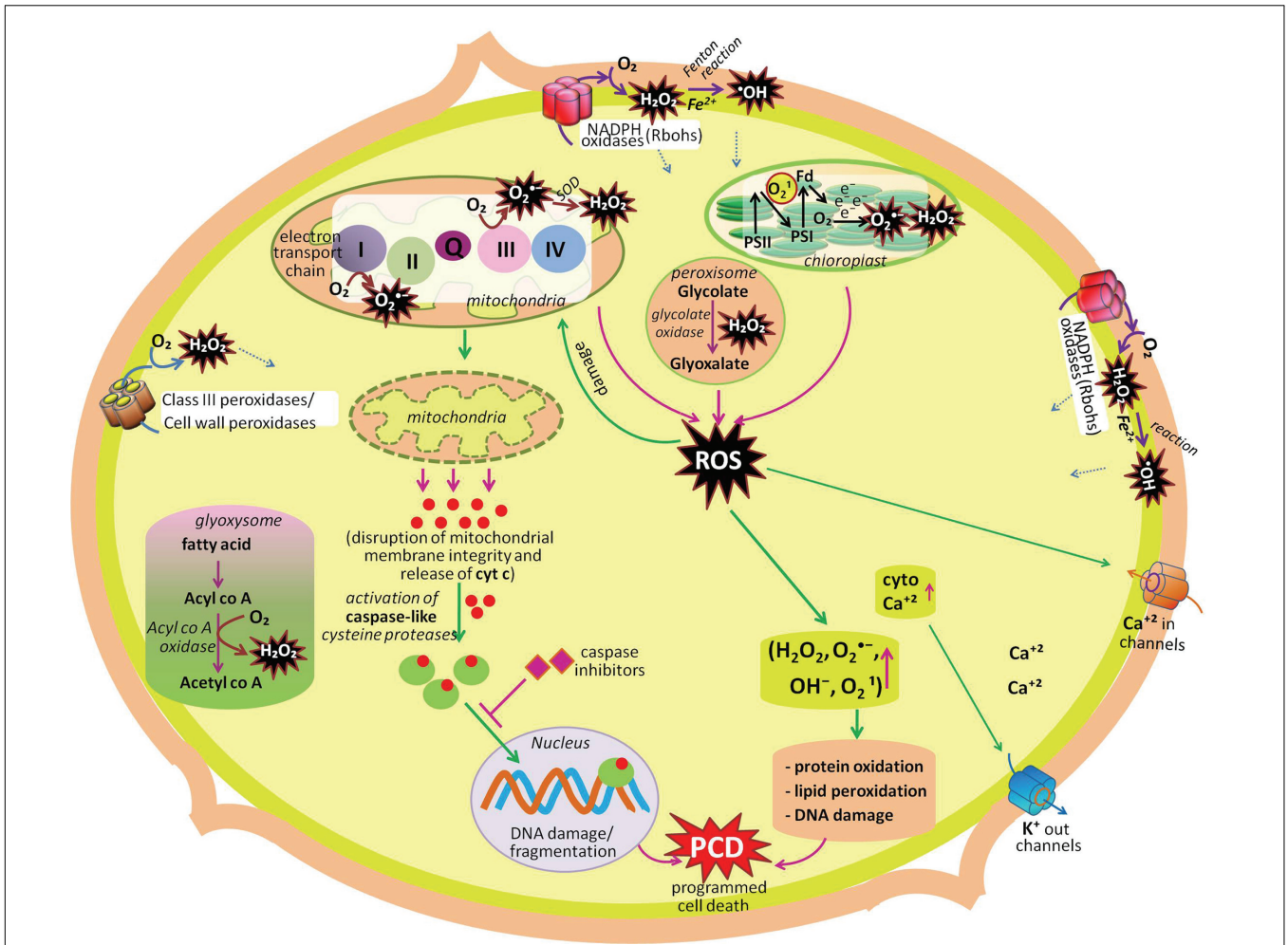

FIGURE 1 | Summary of production and metabolic fate of various ROS (hydrogen peroxide, superoxide radical, singlet oxygen, hydroxyl radical) in different cellular compartments (cell wall, chloroplast, mitochondria, peroxisome, glyoxysome, cytosol, plasma membrane). Among these organelles, chloroplast, mitochondria, peroxisome, and plasma membrane actively participate in developmental programmed cell death (dPCD). Another organelles mentioned in the figure participates in other developmental process of the plant like seed germination, cell proliferation and differentiation, polar cell growth in root hairs and pollen tube, leaf development, etc. (modified after Gadjev et al., 2008; Mittler and Blumwald, 2010; Mittler et al., 2011; Petrov et al., 2015).

of $O_2^{\bullet -}$ (through leakage of electrons from the acceptor side of photosystem II to O_2, from Fe–S centers of photosystem I and reduced ferredoxin (Mehler reaction; Pospisil, 2012), while in non-photosynthetic tissues of plants (i.e., roots, meristems, or seeds) mitochondria are the biggest sources of ROS generation (Navrot et al., 2007; Kalogeris et al., 2014; Huang et al., 2016). In mitochondria, the key sources of ROS production are NADH dehydrogenase complexes I and III, and the ubiquinone pool (Marchi et al., 2012; Steffens, 2014) where $O_2^{\bullet -}$ radicals are generated from the complexes as a by product of energy metabolism by the reduction state of ubiquinone pool (Rhoads et al., 2006; Drose and Brandt, 2012). Other sources of ROS, mainly in non-photosynthetic tissues are NADPH oxidases, cell wall peroxidases, peroxisomes, and glyoxysomes. In glyoxysomes and peroxisomes, H_2O_2 is produced during fatty acid oxidation (by Acyl CoA oxidase), and photorespiration (by glycolate oxidase), respectively (del Rio et al., 2006; Gilroy et al., 2016; Kerchev et al., 2016; Rodríguez-Serrano et al., 2016). In chloroplasts, during impairment of CO_2 fixation, an increased activity of ribulose-1,5-bisphosphate carboxylase/oxygenase leads to the formation of glycolate, which moves to peroxisomes and leads to the formation of H_2O_2, via its oxidation in the presence of glycolate oxidase enzyme (**Figure 1**; **Table 1**). Cell wall-associated extracellular peroxidases and plasma membrane-bound NADPH oxidases (e.g., *Nox*, *Rbohs* or respiratory burst oxidase homologs) are the key enzymes that produce H_2O_2 and $O_2^{\bullet -}$ (usually rapidly dismutated to H_2O_2) in the apoplast (Bindschedler et al., 2006; Sagi and Fluhr, 2006; Choi et al., 2007). In *Arabidopsis*, 10 genes encoding respiratory burst oxidase homologs (Rbohs, i.e., *RbohA-RbohJ*) have been reported (Bedard and Krause, 2007; Marino et al., 2012). Notably, in presence of redox-active metals such as Fe^{2+} and Cu^{+}, H_2O_2

TABLE 1 | Various sites of ROS production and their role in growth and development of plants.

	Sources	Cellular localization	Developmental process	Reference
ROS				
$O_2^{\bullet -}$ and H_2O_2	Electron transport chain	Mitochondria	Seed germination	Moller, 2001; Noctor et al., 2007
$O_2^{\bullet -}$ and H_2O_2	Fatty acid oxidation	Glyoxysome	Seed germination	Huang et al., 1983
H_2O_2	NADPH oxidase	Plasma membrane	Seed germination	Ishibashi et al., 2010
H_2O_2		Peroxisome	Seed germination	Palma et al., 2009
$O_2^{\bullet -}$ and H_2O_2	NADPH oxidase	Plasma membrane	Root growth and root hair development	Foreman et al., 2003
H_2O_2 and $O_2^{\bullet -}$	NADPH Oxidases	Plasma membrane	Pollen tubes growth	Cárdenas et al., 2006
H_2O_2	Class III peroxidases	Cell wall	Leaf development	Lu et al., 2014
dPCD				
$O_2^{\bullet -}$ and H_2O_2	Electron transport Chain, NADPH oxidase	Mitochondria, Plasma membrane	Self-incompatibility in pollen	Wang and Zhang, 2011; Wang et al., 2013
$O_2^{\bullet -}$ and H_2O_2	NADPH oxidase	Plasma membrane	Synergid cells dPCD	Duan et al., 2010, 2014
H_2O_2	NADPH oxidase	Plasma membrane	Tapetal dPCD	Xie et al., 2014
$O_2^{\bullet -}$ and H_2O_2	NADPH oxidase	Plasma membrane	Formation of lysigenous aerenchyma	Rajhi et al., 2011
H_2O_2	NADPH oxidase	Plasma membrane	Secondary wall formation (lignification)	Potikha et al., 1999
H_2O_2	Electron transport chain	Chloroplast	Leaf senescence	Chen et al., 2012
H_2O_2		Peroxisome	Leaf senescence	Palma et al., 2009

will give rise to highly reactive $^{\bullet}OH$ (the most toxic oxidant in the ROS family) or $O_2^{\bullet -}$ via the Fenton or Haber–Weiss reactions, respectively (Jomova et al., 2010).

Parallel to the production of ROS, aerobic organisms have evolved sophisticated and well-outfitted antioxidant defense machinery. This machinery possesses highly efficient enzymatic [superoxide dismutase (SOD); ascorbate peroxidase (APX); catalase (CAT); monodehydroascorbate reductase (MDHAR); dehydroascorbate reductase (DHAR); glutathione reductase (GR); glutathione peroxidase (GPX); glutathione-*S*- transferase (GST); and guaiacol peroxidase (GOPX)] and non-enzymatic [ascorbic acid (ASA); glutathione (GSH); tocopherols, phenolic compounds, and non-protein amino acids] antioxidant defense systems to control over the cascades of uncontrolled oxidation (Gill and Tuteja, 2010) to detoxify ROS in order to balance the cellular ROS level, as the maintenance of redox homeostasis is essential (Singh et al., 2012a,b; Wrzaczek et al., 2013). However, disturbance in the equilibrium between ROS and antioxidant defense system creates a condition of oxidative stress.

Although early research was focused on the toxic nature of ROS, the interest has shifted over the last decade toward their emerging role as signaling molecules in a broad range of physiological processes, such as growth and development, seed germination, programmed cell death (PCD), root growth, and gravitropism (Mittler et al., 2011; Wrzaczek et al., 2013; Baxter et al., 2014). A tight balance between ROS production and scavenging is necessary for the regulatory action of ROS (de Pinto et al., 2012; Sharma et al., 2012; Baxter et al., 2014). The uses of ROS as signaling molecules indicate that plants have evolved the ability to achieve a high degree of control over ROS toxicity (Bhattacharjee, 2012; Mattila et al., 2015). In this review, we have summarized studies from past decade that have improved our understanding about roles of ROS in signaling and the regulation of cellular processes in relation to plant growth and development.

CELLULAR REDOX AND SIGNAL TRANSDUCTION

Coupled oxidation–reduction (redox) reactions in cells are the necessity of life. Various cellular signaling events are mainly based on redox reactions; therefore, it is probable that ROS are directly linked to the cellular redox metabolism. A redox regulatory network is found in each cell whose state is adjusted by ROS and these ROS regulate gene expression, translation, metabolism, and turnover (Dietz, 2016). Accordingly, cell maintains the redox homeostasis by powerful and complex antioxidants such as ascorbate and glutathione and/or antioxidant enzyme systems. Ascorbate and glutathione are much more than simple antioxidants, as they consist of both oxidizing and reducing forms. The antioxidants are principally maintained in the reduced state. A powerful reductant not only favors ROS removal but can also promote ROS generation (Foyer and Noctor, 2016). Alterations in the equilibrium of reduced *vs*. oxidized forms of the antioxidants might be used as a sensor for changes in the environment, and changes in ROS levels which might affect the redox status of the cell. Increased levels of ROS may result into the oxidation of antioxidant systems which change the redox equilibrium of the cell. According to Kleine and Leister (2016) in order to allow appropriate retrograde signaling to the nucleus, coordination of gene expression in between the compartments of the cell needs monitoring of chloroplast, glyoxysome, mitochondrial and peroxisome status. These facts imply that cells have evolved strategies to operate ROS as biological signals that control various developmental programs (Mattila et al., 2015). This explanation is based on the statement that a ROS can interact with a specific target molecule which perceives the elevated ROS concentration, and after that translates this information into a change of gene expression. These changes in transcriptional activity might be achieved by the oxidation of

components of signaling pathways which consequently activate transcription factors (TFs) or directly by modifying a redox-sensitive TF. ROS effects on components of the mitogen activated protein kinases (MAPKs) cascade result in the indirect activation of TFs. In *Arabidopsis*, H_2O_2 activates *Arabidopsis thaliana* MPK6 (AtMPK6) and AtMAPK3 by the activity of MAPKKK *Arabidopsis* NPK1-RELATED PROTEIN KINASE1 (ANP1; Apel and Hirt, 2004) and strongly induces *A. thaliana* NUCLEOTIDE DIPHOSPHATE KINASE2 (AtNDPK2; Banfi et al., 2004). In a recent study, a link between the ROS-response ZAT12 zinc finger protein and iron regulation in cells was explored which suggests that the equilibrium between ROS and iron is crucial for the growth and development of plants preventing the formation of highly toxic $^{\bullet}OH$ (Le et al., 2016). According to Le et al. (2016) ZAT12 interacts and suppresses the function of FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTIONFACTOR, a central regulator of iron deficiency responses. In response to elevated ROS, ZAT12 is up-regulated and suppresses iron uptake thereby preventing the risk of $^{\bullet}OH$ formation. Another example of biological regulatory circuit was reported by Adachi et al. (2015) who have identified a WRKY TF that is phosphorylated by MAPK and a W-box in the promoter region of *Nicotiana tabacum* RBOH, interconnecting the phosphorylation events of MAPK in response to pathogen recognition with the accumulation of RBOH protein. Moreover, in recent years different members of the NAC family of TFs (e.g., Fang et al., 2015; Chen et al., 2016; Zhu et al., 2016), APETALA2/ethylene response TF REDOX RESPONSIVE TRANSCRIPTION FACTOR1 that are regulated by different WRKYs (Matsuo et al., 2015), and different zinc-finger proteins such OXIDATIVE STRESS2 (He et al., 2016) like ROS-response regulatory proteins were identified. Besides TFs, calcium (Ca) waves comprise important components of systemic signaling in plants (e.g., Suzuki et al., 2013; Carmody et al., 2016; Choi et al., 2016; Evans et al., 2016). Several findings summarized that RBOH acts as a central hub in the cellular ROS-signaling network (Baxter et al., 2014; Willems et al., 2016) and this RBOH functions along with MAPK pathways to integrate ROS signals and modulate cell-to-cell signal propagation in local and systemic signaling (Evans et al., 2016; Gilroy et al., 2016). These MAPK pathways take part in retrograde signaling from the chloroplast to the nucleus (Vogel et al., 2014; Dietz, 2016). Thus, MAPK pathways are of fundamental as well as far-reaching importance in converting ROS signals into protein phosphorylation.

ROLE OF ROS IN PLANT GROWTH AND DEVELOPMENT

ROS and Cell Proliferation and Differentiation: A Cascade of Signaling Network

In multicellular organisms, growth mainly depends on the maintenance of an appropriate equilibrium between cell division and differentiation (**Figure 2**). In the case of animals, interruption of this equilibrium between cell division and differentiation can lead to tumoral growth and disease, while in plants; it can lead to premature cessation of organogenesis, or as a consequence of abnormal growth (Zhang et al., 2008). The initial stage of differentiation is marked by the transition from cellular proliferation to elongation, which is regulated by ROS homeostasis (Tsukagoshi et al., 2010). According to Dunand et al. (2007), $O_2^{\bullet -}$ and H_2O_2 are two main ROS, which are differentially distributed within the root tissues of model plant *Arabidopsis*. $O_2^{\bullet -}$ principally accumulates in expanding meristem cells, while H_2O_2 accumulates in the elongation zone (Wells et al., 2010) and an overlap of both types of ROS is observed within the "transition zone" (**Figure 2**). The transition between root cell proliferation and differentiation is mainly controlled by the delicate equilibrium between $O_2^{\bullet -}$ and H_2O_2, which in turn is regulated by a TF: UPBEAT1 (UPB1; Tsukagoshi et al., 2010). The UPB1, a member of the basic/helix loop-helix (bHLH) TF family, shows an increased expression in the root transition zone (Tsukagoshi et al., 2010). Tsukagoshi et al. (2010) have reported that plants over-expressing UPB1 had shorter roots due to a decrease in both meristem size and mature cells, while plants lacking UPB1 (*upb1* mutant) had longer roots with increased meristem size and longer root cells. Furthermore, when Tsukagoshi et al. (2010) investigated the mechanism of action of UPB1 regulated genes, a set of peroxidases (Prxs) was seen to be directly repressed by UPB1. Further investigation pointed out that $O_2^{\bullet -}$ production was reduced in lines over-expressing UPB1 but increased in the elongation zone in the *upb1* mutant, while H_2O_2 was found to be increased in the plants over-expressing UPB1 and reduced in the elongation zone of *upb1* mutant. These findings suggested that the position of the transition zone is determined by the ramp formed by $O_2^{\bullet -}$ in the meristem to maintain the cellular proliferation, and H_2O_2 in the elongation zone, for differentiation. This indicates that UPB1, feedback of which is regulated by H_2O_2, plays a key role in maintaining the balance of $O_2^{\bullet -}$ and H_2O_2 via its control on Prxs expression.

Genetic analysis by Sundaravelpandian et al. (2013) signifies that the subunits of the mediator complex PHYTOCHROME AND FLOWERING TIME1 (PFT1)/MED25 and MED8 are vital for the differentiation of root hairs (**Figure 2**). Moreover, studies have demonstrated that PFT1/MED25 restricts cell growth, while MED8 works independently of PFT1 to control organ growth and regulates ROS balance in roots (Xu and Li, 2011). Transcriptional profiling of roots of *pft1* mutants disclosed that PFT1 triggers H_2O_2 formation mediated by a subset of class III peroxidase enzymes, to maintain the equilibrium between H_2O_2 and $O_2^{\bullet -}$ during root hair differentiation. Perturbed H_2O_2 and $O_2^{\bullet -}$ distribution was seen in *pft1* mutants, suggesting that PFT1 is necessary to maintain the redox homeostasis in roots (Sundaravelpandian et al., 2013).

In the fungus *Neurospora crassa*, at the start of each cell differentiation step, a hyperoxidant state occurs that leads to the development of conidia (Gessler et al., 2007). According to Aguirre et al. (2005), the hyperoxidant state is transient and unstable, and occurs as ROS production exceeds the antioxidant capacity of the cell. It is well-known from this example that antioxidants inhibit developmental processes, which leads to the idea that ROS may be required for these processes in plants as well. In contrast, it is predictable that the deficiency of antioxidant

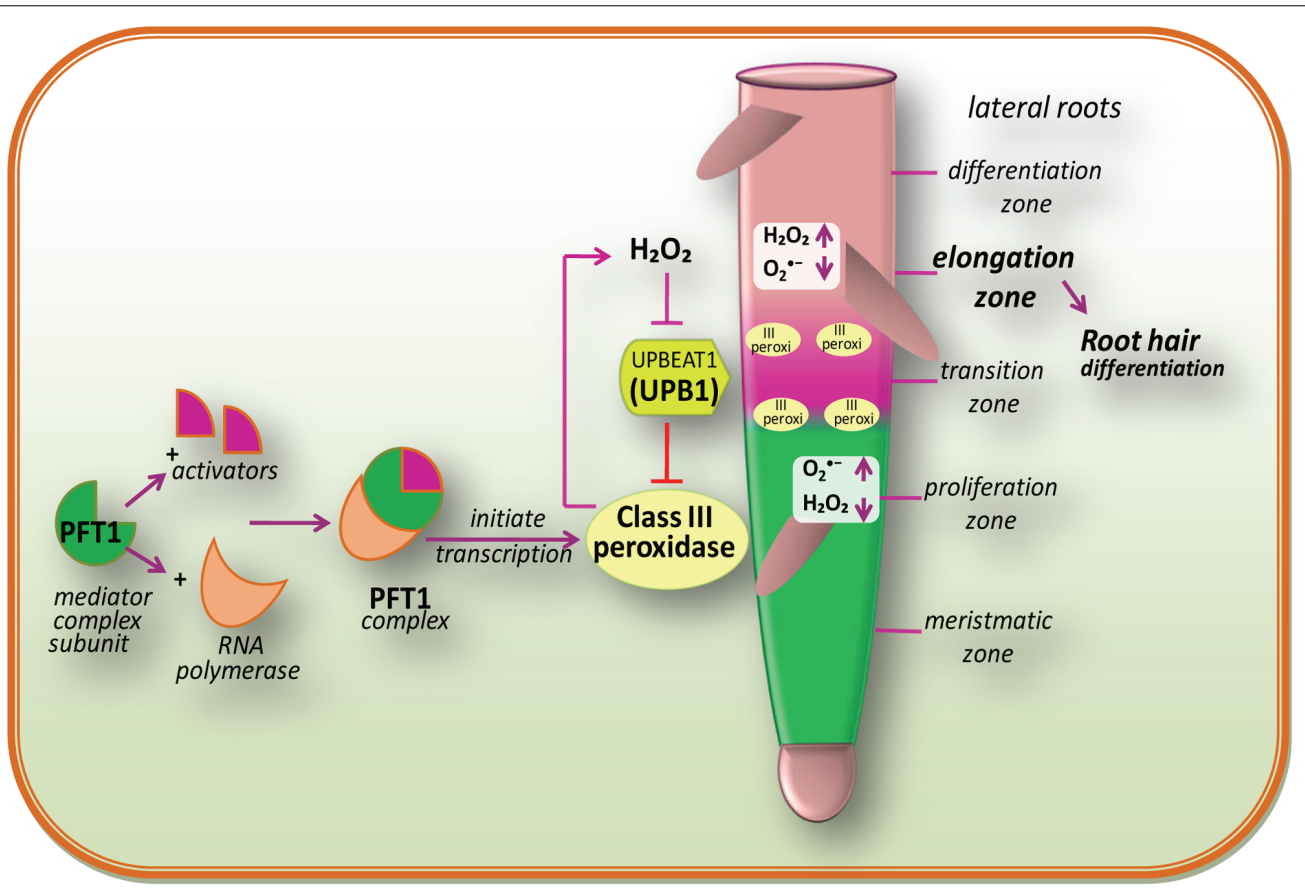

FIGURE 2 | Redox regulation during cell proliferation and differentiation and also a proposed model of PHYTOCHROME AND FLOWERING TIME1 (PFT1)-regulated root hair differentiation (modified after Sundaravelpandian et al., 2013). The PFT1 mediator complex subunit interacts with their activators and RNA polymerase II to initiate the transcription. The PFT1 upregulates class III peroxidases which result into the production of H_2O_2 in the elongation zone while $O_2^{\bullet-}$ is produced by NADPH oxidases in the meristmatic zone. These cellular processes overlap within the "transition zone." A gradient of $O_2^{\bullet-}$ and H_2O_2 in the root controls the transition between cell proliferation and differentiation. The daughter stem cell divides in the proliferation zone, which is characterized by high $O_2^{\bullet-}$ level. Once after reaching into the transition zone, they come across an increased H_2O_2 level and stop dividing and initiate to elongate and differentiate in the differentiation zone. The bHLH transcriptional factor, UPB1, regulates meristem size thereby balancing the $O_2^{\bullet-}/H_2O_2$ ratio by modulating the expression of PEROXIDASE genes (modified after Tsukagoshi et al., 2010).

enzymes will result in to elevated ROS levels, and improved cell differentiation will take place.

ROS and Programmed Cell Death (PCD): An Approach of Life for Plants

Programmed cell death, which is critical in plant organogenesis and survival, is an integral cellular program employed by plants by which targeted cells terminate to demise under certain developmental (vegetative and reproductive) and stress conditions (Aken and Van Breusegem, 2015; Ambastha et al., 2015; Durme and Nowack, 2016). It is essential for removing unwanted diseases or damaged cells, controlling cell number and maintaining the homeostatic balance and thereby improving the endurance of organisms (Petrov et al., 2015; Singh et al., 2015).

Plant PCD is related with a number of developmental processes including embryo formation, degeneration of the aleurone layer in germinating seeds, formation of root aerenchyma and epidermal trichomes, differentiation of tracheary elements, tapetum degeneration, pollen self-incompatibility, floral organ abscission, leaf shape remodeling, and leaf senescence (Gechev et al., 2006). ROS such as H_2O_2 is identified as key modulators of PCD along with other biological processes such as growth and development (Gechev et al., 2006). Concerning developmental programmed cell death (dPCD), a division can be made between (1) differentiation induced PCD that occurs as final differentiation step in specific cell types, for instance, tracheary elements in xylem, the trichome/ root cap, or tapetum layer of anther (Plackett et al., 2011; Bollhöner et al., 2012; Fendrych et al., 2014), and (2) age-induced PCD, i.e., cells must die to form organs for appropriate functions or shapes (unisexual reproductive organs in dicots, aerenchyma tissue, leaf shape), or cells die as they accomplished their function and/or are no longer required (nectaries and petals of flowers after pollination, leaf senescence; Thomas, 2013). Nevertheless, some types of PCDs, including endosperm cell death or pollen self

incompatibility (SI) does not fall into either of above proposed classes (van Doorn et al., 2011).

The life cycle of plant alternates between diploid sporophyte and haploid gametophyte phases. The sporophytic phase starts with zygote (2n) formation and ends with flower bearing adult plant while gametophytic phase starts with sporophytic meiosis and develops into male (pollen grain) and female (ovule) reproductive structures. In dPCD, plants thereby removing tissue or organs which are no longer necessary conserve its energy along with blocking the possible entry sites for pathogens.

According to Tripathi and Tuteja (2007), to protect the initial growth of the ovule, sepals, and petals after accomplishing their role may undergo dPCD, where ROS specifically H_2O_2 is known to be involved in the death of petal cells (Tripathi and Tuteja, 2007). ROS is produced from H_2O_2 therefore, the enzymes regulating H_2O_2 level showed differential expression in dPCD during senescence (Halliwell and Gutteridge, 1989). Panavas and Rubinstein (1998) have also reported an increase in ROS due to increased SOD and decreased CAT activities in daylily plant that results into dPCD.

In megaspore, the chalazal cells differentiated into the three antipodal cells and micropylar cells develop into one egg and the two synergid cells and in center of embryo two polar nuclei are present (Yadegari and Drews, 2004). The synergid cells produce signals which attract and guide the pollen tube growth, ultimately deteriorate in a programmed manner (Punwani et al., 2007). In *A. thaliana*, FERONIA (FER) receptor kinase is responsible for the production of ROS by NADPH oxidase via ROP small GTPase in synergid cells (Duan et al., 2010, 2014) leading to dPCD. ROS by activating Ca^{2+}-permeable channels alter the cell wall extensibility. In *A. thaliana*, ROS production (necessary for pollen tube growth) by the pollen-specific NADPH oxidases: RbohH and RbohJ is mediated by ANX, which maintains a tip-focused Ca^{2+} gradient (Boisson-Dernier et al., 2013; Kaya et al., 2014; Lassig et al., 2014). The excess Ca^{2+} influx might be the reason of ROS-induced pollen tube rupturation/PCD (Duan et al., 2014). Likewise, synergid-derived ZmES4, a defensin-like peptide by opening the K^+ channel induced rapid pollen tube rupturation in maize (Amien et al., 2010). These findings revealed that ROS and other secreted proteins regulate cell wall alteration for the reception of pollen tube through the functions of FER in synergid cells and ANX in pollen tubes. On the opposite end of the ovule, three antipodal cells undergo dPCD immediately before fertilization in mature embryo sacs of *A. thaliana*. It is reported that lifespan of antipodal cell is regulated by central cell so; ROS accumulation specifically in the mitochondria of the central cell might act as a signal in dPCD of antipodal cell in a non-cell autonomous way (Hayashi et al., 2001).

During development of male sex organs, ROS signatures dictate the correct timing of tapetal dPCD because in order to release pollen, tapetum cells must die (Hautegem et al., 2015; Durme and Nowack, 2016). In tapetal dPCD, H_2O_2 acts as a key regulator. In *A. thaliana* RBOHE, a tissue-specific NADPH oxidase is the major H_2O_2 contributor supporting tapetal cell death (Xie et al., 2014). According to Xie et al. (2014) both overexpression and deficiency of RBOHE eliminated usual ROS accumulation leading to the male sterility.

ROS also play a signaling role during self-incompatibility-(SI; to prevent inbreeding by the rejection of incompatible pollen) induced pollen PCD in *Papaver*. According to Wilkins et al. (2011), the simultaneous scavenging of H_2O_2 and NO suppressed SI PCD (Wilkins et al., 2011). SI leads into signal transduction that involves increased ROS, Ca^{2+}, NO, activation of MAPK, and protein phosphorylation as it is supported by large number of evidences that ROS play a key role in SI response that activates dPCD in self-compatible pollen (Bosch et al., 2010; Wilkins et al., 2011; Serrano et al., 2012; Jiang et al., 2014). In poppy, SI is associated with ROS induced activation of Ca^{2+} signaling cascade followed by a release of mitochondrial cytochrome c into the cytosol and caspase-3-like enzyme activity (Bosch and Franklin-Tong, 2007), and process is mediated by the activity of MAPK cascade signaling (Li et al., 2006; **Figure 1**). According to Wilkins et al. (2011, 2015) Ca^{2+} influx boosts H_2O_2 levels to SI-inducing levels that finally triggered a signaling network leading PCD thereby ensuring that fertilization is not achieved by incompatible pollen.

During pollination, when stigma is receptive (ready to receive pollen grains), accumulates high H_2O_2 levels, and that H_2O_2 levels decrease when stigma starts to support pollen development (Zafra et al., 2010; Serrano and Olmedilla, 2012; Serrano et al., 2012). After that, the signal exchanges in between the stigma and the pollen, that might regulate the production of ROS and reactive nitrogen species (RNS) in both tissues (Serrano et al., 2012) takes place. The H_2O_2 level in the stigma which was increased before pollination was found decreased after pollen arrival. Contrary to this, the $O_2^{\bullet -}$ and NO levels were increased with attendant increase in peroxynitrite ($ONOO^-$; Serrano et al., 2015). Treatment with $ONOO^-$ scavengers decreases papillar cell death and also reduces the quantity of pollen grains deploying dPCD, suggesting that ROS mediated PCD signaling takes place during incompatible pollination in the olive (Serrano et al., 2015). After enterance in female gametophyte, pollen tube discharges its content (two male gametes). Out of two male gametes, one fertilizes the binuclear central cell to form a triploid endosperm and other migrates to the egg cell (Yadegari and Drews, 2004). The endosperm functions as a storage tissue for developing embryo which after sometimes undergo ROS induced PCD mediated by abscisic acid (ABA) and ethylene.

During cereal seed germination, gibberellic acid (GA) is produced by plant embryo that activates the aleurone cells to release an amylase enzyme, which sequentially hydrolyzes and mobilizes starch from the endosperm in the seeds thereby providing energy to the embryo. After completion of germination, these aleurone cells are abolished by PCD. According to Yadegari and Drews (2004), ROS and GA play vital roles in the control and implementation of aleurone PCD (discussed in next section).

Besides embryonic development, ROS also play a key role in dPCD of vegetative parts; the development of xylem tracheary elements is one of them. Contrary to other cell type in plants, xylem tracheary elements are functionalized by PCD leading into hollow cell cadavers with merely a remaining cell wall demarcating the sap conducting cylinder (Roberts and McCann, 2000). H_2O_2 by increasing cross-linking of polymers induces

stiffening of the cell wall as growth ceases next to onset of differentiation (Hohl et al., 1995; Schopfer, 1996). Lignification in stem of *Zinnia elegans* L. is characterized by a burst in H_2O_2 production, which could act as a developmental signal in secondary wall differentiation (Potikha et al., 1999). This H_2O_2 may coordinate and regulate at the transcriptional level mRNAs synthesis encoding phenylalanine ammonia-lyase like lignin biosynthetic enzyme (Desikan et al., 1998), and xylem peroxidases (Wu et al., 1997) or trigger xylem differentiation thereby inducing PCD (Fukuda, 1996) and formation of secondary cell wall. The major H_2O_2 production site in the differentiating xylem is outer-face of the plasma membrane of both non-lignifying xylem parenchyma cells and differentiating thin-walled xylem cells. From their sites of production, H_2O_2 diffuses (mainly through the continuous cell wall space) to the differentiating (cells which are at the state of secondary cell wall-formation) as well as differentiated xylem vessels (cells already have completed secondary cell wall formation, i.e., lignified; Ros Barceló, 2005), and H_2O_2 is thought to not limit the rate of xylem lignification (Ros Barceló, 2005). These findings strongly supported that H_2O_2, which is a key component for inducing dPCD signaling network in lignifications of xylem vessels, is mainly produced from non-lignifying xylem parenchyma cells as it is necessary for the polymerization of cinnamyl alcohols.

A similar picture has been reported in various forms of developmental cell death— formation of aerenchyma and the senescence-associated cell death. According to del Rio et al. (2006), ROS like $O_2^{\bullet -}$ and H_2O_2 are the key coordinators of senescence (Lam, 2004). In the internodes of rice, the exogenous application of H_2O_2 promotes the formation of lysigenous aerenchyma (Steffens et al., 2011). In cortical cells of maize roots, Rajhi et al. (2011) have reported a strong up-regulation of gene encoding RBOH, thereby suggesting that RBOH mediated ROS generation participates in the formation of lysigenous aerenchyma.

In many plants, trichomes in their fully differentiated stage are dead. The development of trichome trails a switch from mitosis to endoreduplication, branching of cells, expansion, and ultimate cell death initiated by H_2O_2 burst (Hulskamp, 2004). In trichomes of succinic semialdehyde dehydrogenase (key enzyme of γ-aminobutyrate metabolic pathway) knockout plants, enhanced H_2O_2 levels are observed (Bouche et al., 2003). In the stem nodes of rice plants, the epidermal cells that cover the primordia of adventitious roots undergo cell death before the emergence of adventitious root. Ethylene promotes epidermal cell death, which is mediated by an excess H_2O_2 production (Steffens and Sauter, 2009). In rice plant, a non-enzymatic H_2O_2 scavenger metallothionein *MT2b* when downregulates it promotes ROS level in cell (Wong et al., 2004). In case of epidermal cells, ethylene downregulates *MT2b* that results into PCD thereby revealing that ethylene promotes ROS accumulation that lead to *MT2b* mediated cell death (Steffens and Sauter, 2009).

Significant roles of ROS have also been reported during natural course of senescence, the last step of leaf development in the life span of an annual plant (Lee et al., 2012; Springer et al., 2015). The accumulation of ROS have been suggested to be an age-associated factor that triggers leaf senescence (Khanna-Chopra, 2012). Foyer and Noctor (2005) proposed that ROS-triggered senescence is not caused by physicochemical damage of the cell, but that these molecules (ROS formation due to leakage of electrons from ETC to O_2 at the onset of senescence) behave as signals, which activate gene expression pathways leading to suicide events (Sandalio et al., 2013; Jajic et al., 2015). During cell senescence, lipid peroxidation can be activated either by lipoxygenases in some tissues, where the activity of lipoxygenase increases with increasing senescence, or by ROS (Chopra, 2012; Bhattacharjee, 2014). Therefore, it was concluded that, during senescence, lipoxygenase plays an essential role in promoting oxidative damage, in which not only stimulates lipid peroxidation but it can also form $^1O_2$. Vanacker et al. (2006) have also reported that in senescent leaves, H_2O_2 content increases in parallel with an increase in lipid peroxidation and protein oxidation. So it is obvious that H_2O_2 plays a crucial role in the regulation of senescence signaling.

After senescence, the resulting plant organ detaches from main plant body and the process is called abscission. In several studies, it has been reported that Prxs are regularly expressed during diverse types of abscission (Meir et al., 2006; Cai and Lashbrook, 2008).

Before discussing the role of ROS in leaf abscission, the notion should be recall that Prxs actively participate in the cell wall loosening (Schweikert et al., 2000; Schopfer et al., 2001). Sakamoto et al. (2008) demonstrated that continuous H_2O_2 production in the cell wall participates in leaf abscission signaling, which is needed to induce the expression of cellulase enzyme (the cell wall-degrading), and when this continuous H_2O_2 production was suppressed by ROS inhibitors, it inhibited the cellulase activity and consequently inhibited abscission. On the other hand, exogenous application of H_2O_2 enhanced cellulase expression and abscission therefore, signifying that H_2O_2 production is essential to induce abscission. After continuous cellulase production (induced by H_2O_2), enhanced H_2O_2 generation at the Abscission-Zone in the Abscission-Zone-separating period was observed that might play a role in the cleavage of wall polysaccharides and loosen the cell wall (Gapper and Dolan, 2006). Therefore, a huge ROS production that probably driven by Prxs at the late stage of abscission may be associated with the cell wall degradation process.

ROS in Seed Dormancy and Early Seed Germination: A Hub of Regulatory Networks

Seed dormancy and seed germination mechanisms are essential processes in plant development, and are believed to be a part of a complex regulatory network (**Figure 3**). In this network, ROS are considered to be key signaling actors as reported in *A. thaliana* (Liu et al., 2010; Leymarie et al., 2012), barley (Ishibashi et al., 2010; Bahin et al., 2011), cress (Müller et al., 2009), wheat (Ishibashi et al., 2008), and sunflower (Oracz et al., 2007). Seed germination starts with water uptake, along with

initiation of cell division, and ends with radicle protrusion (Holdsworth et al., 2008; Bewley et al., 2013). In dry seeds, due to highly reduced enzyme activities, ROS are probably originated from lipid peroxidation like non-enzymatic reactions that occur even at very low moisture contents (Vertucci and Farrant, 1995; McDonald, 1999). However, in hydrated seeds (that occurs following imbibition) ROS may generate from all metabolically active compartments like chloroplasts (by election transfer in photosystems), glyoxysomes (by lipid catabolism), mitochondria (via respiratory activity), peroxisomes (by purine catabolism), and plasma membranes (by NADPH oxidase; Bailly, 2004). In hydrated seeds, the continuation of respiration (in mitochondria) in imbibed seeds can lead to electron leakage because the electrons of mitochondrial electron-transfer chain (mETC) have sufficient free energy to directly reduce the O_2, and this can be considered as unavoidable source of increased ROS production in mitochondria (Rhoads et al., 2006; El-Maarouf-Bouteau and Bailly, 2008). Due to this reason, in hydrated seeds, the mitochondrial activity is considered as major source of ROS (such as H_2O_2) production during germination (Noctor et al., 2007). Moreover, in hydrated seeds, ROS targets may be close or far from their production sites as the free water allows ROS (or their longer-living or movable forms like H_2O_2) to move and reach their targets farther from their production sites while, in dry seeds the ROS targets must be close to their production sites (Bailly et al., 2008). It has been reported that H_2O_2 is produced in the seeds of *Zinnia elegans* (Ogawa and Iwabuchi, 2001), maize (Hite et al., 1999), wheat (Caliskan and Cuming, 1998) and soybean (Puntarulo et al., 1988) during the early imbibitional period, and cellular responses to H_2O_2, which is the most labile ROS messenger, mostly rely on the involvement of redox-active proteins (Foyer and Noctor, 2013), stimulation of redox-sensitive TFs that orchestrate downstream cascades (Petrov and Van Breusegem, 2012), oxidation of specific peptides (Moller and Sweetlove, 2010), and the activation of MAPKs (Barba-Espín et al., 2011). ROS produced during imbibition in different sub-cellular compartments, largely affect the expressions of various genes (Neill et al., 2002). The sub-cellular ROS interact with specific target molecules (particular for increased ROS concentration), which there translate that information by altering the gene expression (Laloi et al., 2004). Oxidation of certain components that keep on to activate TFs of the signaling pathway, or the direct modification of redox-sensitive TFs by ROS, may led to the alteration of transcriptional activity (Laloi et al., 2004).

It has been reported in several studies that during seed imbibition, ROS oxidize specific proteins for driving the germination process (Barba-Espín et al., 2011; El-Maarouf-Bouteau et al., 2013), or oxidize mRNAs to prevent their translation (Bazin et al., 2011).

According to El-Maarouf-Bouteau and Bailly (2008), ROS have well-recognized role in endosperm weakening, protection against pathogens, mobilization of seed reserves, and PCD in seeds. In some seeds, an endosperm covers the radical tip and acts as a mechanical barrier in radicle protrusion.

When addressing the event of seed germination and seedling establishment, it appears exceedingly relevant to consider and investigate the possible interaction between ROS and plant hormones in the process of germination, as ROS involving PCD process have been established in aleurone layer of cereal grains, which are closely related to H_2O_2 interactions with GA and ABA hormones (Ishibashi et al., 2013; Corbineau et al., 2014; Miransari and Smith, 2014; **Figure 3**). It is known that in aleurone cells of cereals, GA supports germination as well as post-germination processes, which are inhibited by ABA. Although GA downregulates the ROS scavenging enzyme activities, they are retained by ABA, which advocates a ROS link to PCD execution. *In vivo*, ROS can damage/oxidize/weaken the cell wall polysaccharides, activate calcium channels, Ca^{2+} binding proteins, MAPK cascades, and damaged cell wall polysaccharides, permit elongation of the growing radicle, which is a characteristic feature of endosperm rupturation in the aleurone layer (Müller et al., 2009; Xu et al., 2010; Barba-Espín et al., 2011; Diaz-Vivancos et al., 2013).

These authors showed that wall polysaccharides are oxidized *in vivo* by the developmentally regulated action of apoplastic $^{\bullet}OH$. The cell wall loosening is assumed to involve the enzymatic as well as non-enzymatic cleavage of structural polysaccharides (Passardi et al., 2004). The regulated action of apoplastic $^{\bullet}OH$ radical, which is generated by Prxs and/or the Fenton reaction in the cell wall (reaction 1) of radicles and endosperm caps, is known to cause the cleavage (breakdown) of hyaluronate (Stern et al., 2007), chitosan (Tanioka et al., 1996) and pullulan-like polysaccharides (Crescenzi et al., 1997).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-} \quad (1)$$

The production as well as the action of $^{\bullet}OH$ increased during endosperm weakening and radicle elongation, and were inhibited by ABA. These outcomes were reversed by GA, representing a positive role of $^{\bullet}OH$ in cell wall loosening during seed germination (Müller et al., 2009).

When aleurone cells are exposed to GA, α-amylase and other hydrolytic enzymes are synthesized and secreted. Contrary to this, antioxidants (activities of CAT, APX, and SOD) suppressed the induction of amylases. While ABA acts as an antagonistic factor blocking the GA response in aleurone cells via the expression of a different set of genes- reinforcing the idea that H_2O_2 functions in GA and ABA signaling thereby regulating α-amylase production in aleurone cells (Nonogaki, 2014).

In seeds, a majority of studies have reported that ABA suppresses ROS generation, i.e., it acts as a negative regulator during seed germination (Ishibashi et al., 2012; Ye et al., 2012) while behaves as a positive regulator of the induction of dormancy (Finkelstein et al., 2008). In presence of GA, exogenous H_2O_2 had minor effect on the degradation of primary transcriptional repressor, i.e., DELLA proteins Slender1 (SLN1) mediating GA signaling, but it supported the production of the mRNA encoding GAMyb (GA-regulated Myb TF) that acts in downstream of SLN1 protein and engages α-amylase mRNA induction. Moreover, H_2O_2 holdbacks the production of ABA-responsive protein kinase (PKABA) mRNA, which is induced by ABA; the production of GAMyb mRNA is repressed by PKABA (Ishibashi et al., 2012). From these studies,

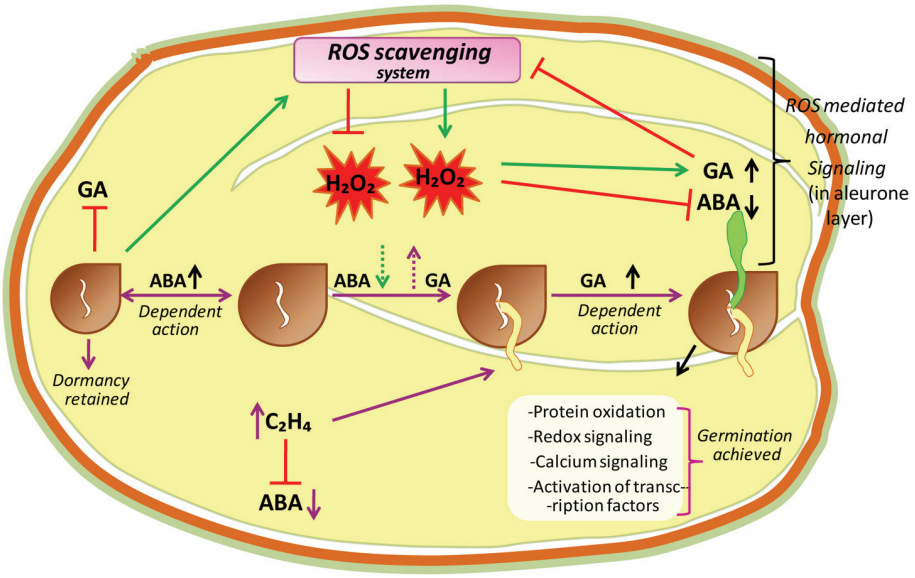

FIGURE 3 | A hypothetical model proposing central role of ROS in the mechanisms of seed dormancy release and germination. The ROS core plays a central in the regulation of germination through the crosstalk with abscissic acid (ABA) and ethylene (C_2H_4). In dormant state, high amount of ABA maintains dormancy by inducing an active signaling pathway. In non-dormant seeds, ABA concentration lowered which is associated with higher ROS level. Apparently H_2O_2, which could in turn interfere with ABA and GA signaling pathways, modify redox status and induce protein oxidation, redox signaling, calcium signaling and transcriptional factor activation leading to the germination of seeds.

Ishibashi et al. (2012) have concluded that the repression of GAMyb mRNA was released by H_2O_2 via PKABA and this H_2O_2 subsequently promoted the production of α-amylase mRNA, thereby suggesting that GA generated H_2O_2, act as a signal that antagonizes ABA signaling in aleurone cells.

Ethylene is another hormone that acts as a positive regulator of seed germination. In soybean seeds, Ishibashi et al. (2013) observed that during seed imbibition ROS are produced, which further promotes ethylene production in sunflower seeds. Recently, Arc et al. (2013) have reported that the interaction between ABA and ethylene have antagonistic effects during seed germination. Moreover, by giving special consideration to cross-talk between ABA, ethylene, and ROS, mechanisms of action of ROS in the process of germination have been elucidated (**Figure 3**).

According to Barba-Espín et al. (2011) ROS also participate in breaking seed dormancy and in germination by activating the oxidative pentose phosphate pathway (oxPPP). The authors observed that the exogenous application of H_2O_2 induces the carbonylation of enzymes taking part in restoration of reducing power during glycolysis, which stimulated oxPPP. Moreover, a relationship between H_2O_2 contents and gene expression related to MAPKs has also been noticed by Barba-Espín et al. (2011). According to Xu et al. (2010) during seed germination, MAPK cascades affect cell division and the hormone so, Barba-Espín et al. (2011) pointed out that variations in H_2O_2 contents (increases during germination) of seed may induce germination thereby activating those MAPK cascades.

ROS and Root Gravitropism

After seed germination, the part of seedling which have to make root and shoot, sense the direction of the gravity vector and elicit gravitropic growth, generally sending the roots to downward and shoot to return to upward and directed growth. Generally it is accepted that growth responses reveal differential auxin redistribution to the flanks of the organ that results into reduction of elongation on one side and acceleration on the other. This differential growth in response to gravity leads to the bending of organ. These gravitropism responses have been connected to ROS generation in the roots of *Arabidopsis* and maize (Joo et al., 2001, 2005). In gravistimulated roots, ROS accumulation was found asymmetrical to the lower cortex within 30 min of reorientation, which becomes symmetrical upon longer stimulation (Joo et al., 2001). Asymmetrical application of H_2O_2 impregnated agar block causes root bending toward block. Here, some caution must be exercised such as H_2O_2 application will also interact with the wall that might have profound effect on cell extensibility. ROS are well-known to play a critical role in cell integrity that might also potentially lead to growth arrest. However, treatment with *N*-acetyl cysteine (NAC)-like ROS scavengers was observed to inhibit the response or curvature without affecting growth rate thereby suggesting that asymmetrical ROS generation is responsible for the gravitropic response (Joo et al., 2001). As the gravitropic responses are known to originate from inhibition of root elongation on the lower flank of the root, these findings are reliable with ROS accumulation to the lower flank acting as a growth inhibitor.

It was thought that auxin might be a primary regulator of such kind of root elongation where it is accumulated on the lower flank to inhibit the growth of gravistimulated root. Consistent with these thoughts, Schopfer et al. (2002) reported that auxin can evoke ROS generation in a process which shows a close parallels to the animal model of ROS generation via NAPDH oxidases (Joo et al., 2005). A similar story is also emerging of a potential role of ROS in the gravitropic response of the maize roots. The role of ROS in auxin-induced root gravitropic responses has been reported in *Zea mays* (Joo et al., 2001), and results strongly suggest that ROS-mediated auxin redistribution by gravity stimulates an increase in gravitropic curving. Firstly, Joo et al. (2001) noticed that ROS are formed subsequent to gravitropic stimulus. When maize roots were kept horizontally, the ROS generation was detected in the apex, stimulating the gravitropic response (Joo et al., 2001). Secondly, ROS production is increased by auxin application to root cells in intact plants as well as in root protoplast cultures (Cervantes, 2001). Further treatment of roots with *N*-1-naphthylphthalamic acid (NPA, an auxin transport inhibitor) results in inhibition of gravitropism and the effect of NPA can be reversed by adding H_2O_2 which has indicated a direct causal relationship between auxin, ROS, and gravitropism (Cervantes, 2001). It has been observed that root bending, after pretreatment with the auxin transport inhibitor NPA, was brought about by unilateral ROS application to vertical roots. However, ROS scavenging by the antioxidants NAC, trolox and ASA inhibits root gravitropism (Joo et al., 2001). However, the effect of NPA was reversed by addition of H_2O_2. Joo et al. (2001) pointed out that ROS might work by kinase activation, but also considered that other mediators of the gravitropic response, such as inositol (1,4,5)-trisphosphate and calcium, might be involved. Furthermore, Joo et al. (2005) observed that phosphatidylinositol 3-kinase is involved in auxin-mediated ROS production that regulates root gravitropism while retreatment with the phosphatidylinositol 3-kinase activity inhibitor LY294002 blocked the auxin- mediated ROS generation, decreasing the sensitivity of root tissue to gravistimulation (Joo et al., 2005).

It is well-known that in plant gravitropism, redistribution of auxin plays an important role (Petrasek and Friml, 2009) in ROS mediated root gravitropism. To find out the role of auxin and ROS, Lupini et al. (2013) used the auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and naphthylphthalamic acid (NPA) and a secondary metabolite coumarin which are known to alter the gravitropic responses in the roots of *A. thaliana*. Coumarin itself did not show any change in gravitropic response, but when added with NPA or TIBA, it reversed the effect of inhibitors (Lupini et al., 2013). Moreover, Lupini et al. (2013) studied ROS distribution in root tips and reported that NPA or TIBA causes distribution of $O_2^{\bullet -}$ around the root tip, which disappeared after coumarin addition to both treatments, restoring ROS localized distribution. These findings suggest that coumarin effect in restoring the root curvature did not depend upon auxin redistribution, but was mediated by ROS generation. Further to investigate the localization of ROS, Libik-Konieczny et al. (2015) by using blue formazan (NBT; precipitates $O_2^{\bullet -}$ accumulation) and 3,3′-diaminobenzidine (DAB; for H_2O_2 accumulation) demonstrated that $O_2^{\bullet -}$ was localized within the tip of root primordia, vascular cylinder cells as well as in the distal and middle parts of newly formed organs during the early stages of rhizogenesis; while H_2O_2 was pronounced in cortical and vascular bundle cells. On adding DPI to the medium, $O_2^{\bullet -}$ accumulation was then restricted to epidermis cells, while that of H_2O_2 was limited in vascular tissues (Libik-Konieczny et al., 2015). These findings suggest that $O_2^{\bullet -}$ engages itself in the process of rhizogenesis, while H_2O_2 is engaged in developmental processes such as cell growth.

ROS in Growth and Development of Root Hair and Pollen Tube

In plant system, the tip growth in either of the cases is mainly associated with the deposition of membrane and new wall materials centered toward the apex of elongating cells, thereby resulting into the formation of tube-like structure. To produce a correct shape and size, cell expansion needs to be carefully regulated. Polar cell growth is continued by oscillatory feedback loops including three main components, i.e., ROS, Ca^{2+} and pH that together play an important role in regulating this process over time (Mangano et al., 2016). Apoplastic ROS balance controlled by NADPH oxidases and class III peroxidases has a great impact on cell wall properties during cell expansion (Mangano et al., 2016).

During expansion of polar cell (root or pollen), the apical zone is characterized by a tip-focused elevated cytoplasmic Ca^{2+} ($_{cyt}Ca^{2+}$) gradient and related proteins along with the apoplastic ROS ($_{apo}$ROS) generation (Konrad et al., 2011; Qin and Yang, 2011; Hepler et al., 2012; Steinhorst and Kudla, 2013). Cell takes Ca^{2+} either from external sources or stored in the cell wall and this Ca^{2+} released by changing $_{apo}$pH which is mainly controlled by plasma membrane located activation/deactivation of H^+ pumps (AHA). Additionally, Ca^{2+} can be stored in vacuoles and ER-Golgi and released into the cytosol. To maintain low concentrations of $_{cyt}Ca^{2+}$, autoinhibitory P-type IIB Ca^{2+}-ATPases (ACAs) transport Ca^{2+} back to the apoplast. In addition to this, the H^+/Ca^{2+} antiporter activity of calcium-exchanger (CAX) translocates Ca^{2+} back to the apoplast and, at the same time, imports H^+ into the cytoplasm. In the tip zone, high levels/concentration of $_{cyt}Ca^{2+}$ activates $_{apo}$ROS generation by NADPH oxidases (NOXs). Moreover, high levels of ROS quickly elevate the concentration of $_{cyt}Ca^{2+}$ (Foreman et al., 2003; Duan et al., 2014) by an unknown phenomenon. NOXH/NOXJ (Wu et al., 2010; Boisson-Dernier et al., 2013) and NOXC (Foreman et al., 2003; Monshausen et al., 2007, 2008) were previously proposed to connect $_{apo}$ROS production with the rapid activation of Ca^{2+} channels (CaCs) of plasma membrane in growing pollen tubes and root hairs, respectively. The idea that ROS are important for sustaining polar growth came into existence with the cloning of root hair defective 2 (*rhd2*) mutants of *A. thaliana* (Foreman et al., 2003). In *rhd2* mutant background, according to Monshausen et al. (2007) and Macpherson et al. (2008) root hairs correctly initiated their developmental program and form bulges on the epidermal cell side but they fail to elongate/transition to tip growth.

When alleles of the *rhd2* were cloned, they were found to inhabiting a gene encoding the respiratory burst oxidase homolog C (AtRBOHC) of *Arabidopsis*, an NADPH oxidase similar to mammalian gp91phox (The phox91 family is conserved throughout the animal, plant and, fungal kingdoms; Foreman et al., 2003), responsible for ROS production during oxidative burst. It means that RHD2 plays an important role in electron transfer from NADPH to an electron acceptor, and resulting in ROS formation. Besides RHD2, another target of ROS signaling in growing root hair has been reported, i.e., OXIDATIVE BURST INDUCIBLE1 (OXI1) kinase (Rentel and Knight, 2004). OXI1 is a Ser-Thr kinase, which plays a role in elongation of root hairs, as it is slightly shorter in *oxi1* mutant than wild type. It was suggested that RHD2-mediated ROS leads to the activation of a MAPKs cascade through OXI1 during growth of root hairs.

A ROS burst is crucial for the rupturation of pollen tube and sperm release (Duan et al., 2014). Oscillatory growth is also related to changes in pH which is regulated by cation (H^+)/anion (OH^-)-permeable channels, by membrane H^+-ATPases (AHA; Falhof et al., 2016) and antiporters (Ca^{2+}/H^+ exchangers). AHA is supposed to be responsible for pumping H^+ out of pollen tubes remains to be identified while AHA2 is highly expressed in growing root hairs. AHA directly controls apoplastic pH ($_{apo}$pH), which affects the enzymatic mechanism that alters cell wall components during cell expansion. Polar growth entails the stretching and deformation of the existing wall in the apical zone, which is escorted by the secretion of new wall materials (Altartouri and Geitmann, 2015). Oscillations in Ca^{2+}, ROS and pH are coupled to transient cell wall loosening to facilitate turgor-driven localized cell expansion (Braidwood et al., 2014; Spartz et al., 2014; Wolf and Höfte, 2014). In cells, growth is determined by the balance between cell wall loosening and stiffening. Root hair growth has to occur not only in accordance with the loosening of the cell wall to allow the expansion at its tip, but also with a need to avoid tip bursting by maintenance of wall integrity. In order to attain this pattern, the factors engaged in pollen tube growth such as $_{cyt}Ca^{2+}$ levels, pH, and NADPH are found to oscillate in such a manner in which the highest concentration follows the highest peak in growth, in concert to maintain the periodic oscillations (Cárdenas et al., 2006; Lovy-Wheeler et al., 2006). In root hairs, the maximum of these oscillatory fluctuations in $_{apo}$ROS concentration and apoplastic/cytoplasmic pH lead cell growth peaks by 7–8 s, while $_{cyt}Ca^{2+}$ oscillations delay oscillations in cell growth by approximately 5–6 s (Monshausen et al., 2007, 2008). In pollen tubes, the oscillations in $_{cyt}Ca^{2+}$ concentration are lagged by approximately 11 s in relation to cell expansion rates (Pierson et al., 1994). Thus, polar cell growth is improved, and perhaps rapidly repressed, by high concentration of $_{cyt}Ca^{2+}$ and, consequently, high $_{apo}$ROS concentrations and a more alkaline $_{apo}$pH.

In *Arabidopsis*, by using high-resolution growth measurements, Monshausen et al. (2008) showed that the frequency of growth pulses was approximately 3 per minute, similar to the frequency of pulses of ROS production and to the oscillations in the apical Ca^{2+} gradient. A comprehensive study of these oscillations implied a growth burst at 5 s due to an increase in tip-focused Ca^{2+} that may be mediated by a stretch-activated Ca^{2+} channel, which is followed 2 s later by ROS production in the wall (Monshausen et al., 2008). According to Monshausen and Gilroy (2009), this ROS production may play a role in covalent cross linking of polymers, and therefore strengthen the wall to prevent expansion and bursting. Monshausen et al. (2007) reported that tip growth ceased after the addition of external H_2O_2; however, scavenging of ROS promoted tip bursting, which suggests that ROS also play a role in wall rigidification. From these facts, it can be concluded that ROS-activated Ca^{2+}-permeable channels induce Ca^{2+} influx to increase the $_{cyt}Ca^{2+}$ after the beginning of Rboh-mediated ROS generation, which further activates Rbohs. Therefore, Ca^{2+} increases in the cytoplasm probably response to increase ROS that alter Ca^{2+} influx.

If we are talking about ROS signaling, it is noteworthy that changes in extracellular pH correlate with the oscillations in growth, which permit the alteration of tip growth in root hairs of *Arabidopsis* (Monshausen et al., 2007). The stiffening of the cell wall is carried out by both ROS and increased pH, which make cell wall resistant to turgor pressure. In the growing tip, oscillation of Ca^{2+} gradients is followed by alkalinization peaks in the apoplast along with the constitutive $O_2^{\bullet -}$ generation across the subapical part of the tip (Monshausen et al., 2009; Swanson and Gilroy, 2010). An oscillatory component of extracellular pH at the tip of pollen tubes has been reported by Messerli and Robinson (2007), that changes the phase by producing ROS and increases the growth by increasing the Ca^{2+} accumulation (Messerli and Robinson, 2007). A similar series of events ROS, pH, Ca^{2+}, and growth have also been reported in root hair tips (Monshausen et al., 2007). These findings put forward that one factor (either ROS or wall pH) compensate with at wall dynamics as well as in controlling the cytosolic activities needed to maintain the growth (**Figure 4**).

Lateral root development is controlled by the antagonistic action of auxin and ABA. Auxin promotes the separation of pericycle initials and cell expansion while, ABA is essential during each stage of lateral root growth as it regulates the balance between cell proliferation and differentiation in the root meristem and in lateral root primordia (Dubrovsky et al., 2008; De Rybel et al., 2010; Lavenus et al., 2013). Both hormones also mediate ROS accumulation in growing roots and promote cell expansion. A transcriptomic analysis (Manzano et al., 2014) of roots treated with auxin revealed that majority of the peroxidase genes in the cs-SKP2B (SKP2B is a marker for lateral root development while cs-SKP2B dataset is its specific expression pattern of cell sorting that regulates the developmental program endogenously) dataset are not regulated by ABA. Tsukagoshi et al. (2010) proposed that for growth regulation at root meristem, Prxs activity (regulated by *UPB1*) acts independently of the auxin pathway. In contrast, Ma et al. (2014) advocated that the auxin response involves ROS signaling. Peroxidases take part in lignin formation in the primary cell wall (Marjamaa et al., 2009). It is notable that indole-3 acetic acid (IAA) is easily degraded by plant Prxs (Cosio et al., 2008), which leads to the reduction of auxin pools. In tobacco plant, free IAA

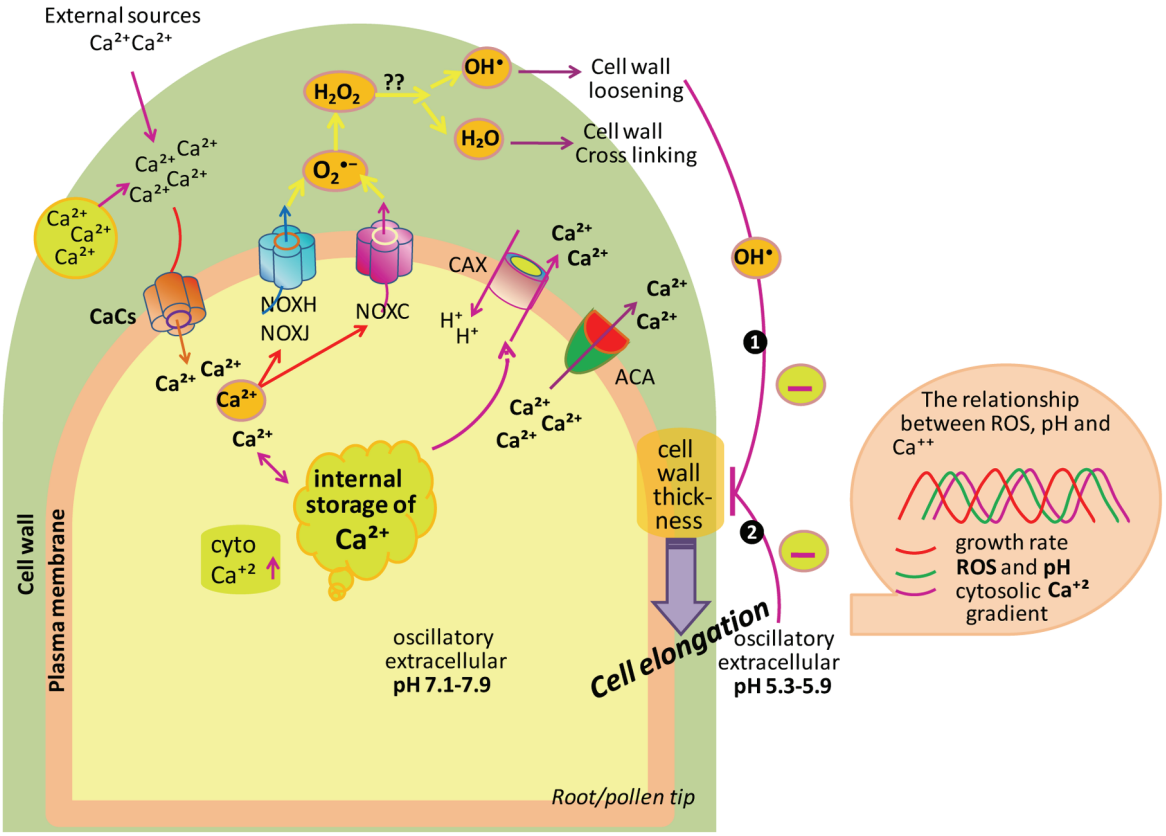

FIGURE 4 | Model depicting the role of ROS, Ca^{2+} and pH in tip growth of root/pollen cells. Cell uptakes Ca^{2+} from its surrounding. To maintain the low levels of $_{cyt}Ca^{2+}$, ACAs transport Ca^{2+} back to the apoplast. Besides this, the H^+/Ca^{2+} antiporter translocates Ca^{2+} back to the apoplast and, at the same time, imports H^+ into the cytoplasm. The $_{cyt}Ca^{2+}$ activates NOXC and NOXH/NOXJ to produce $_{apo}$ROS in root hairs and pollen tubes, respectively. NOX produces apoplastic $O_2^{\bullet -}$, which is dismutated by SOD to H_2O_2. Also, $_{apo}H_2O_2$ and O_2 generate $^{\bullet}$OH (in the hydroxylic cycle) which catalyzes the nonenzymatic cleavage of polysaccharides, thereby allowing tip growth. Box on right side showing that extracellular ROS, pH as well as $_{cyt}Ca^{2+}$ are coupled to growth oscillations. (Modified after Monshausen et al., 2009; Mangano et al., 2016).

levels decreased in roots, indicating that alteration of auxin level is critical for the inhibition of lateral root formation in plants (Moriwaki et al., 2011). Recently, it was proposed that root architecture is determined by the redox-mediated GPX family (Passaia et al., 2014). Moreover, Passaia et al. (2014), through genetic analysis, showed that *gpx* mutants differentially affect lateral root formation (except *gpx3-2* mutants), with all *gpx* mutants showing expanded lateral root primordia (LRP). Passaia et al. (2014) have also observed that *GPX1* and *GPX7* are the two main Prxs to play a significant role in the regulation of lateral root development and root architecture, which is also associated with auxin-dependent control of lateral root formation. In addition, Manzano et al. (2014) have shown that H_2O_2 and $O_2^{\bullet -}$ actively participate in lateral root development. They have also reported that in the cs-SKP2B dataset, several enzymes are involved in ROS formation, including AtrbohC, cytochrome P450 electron carrier proteins, and lipoxygenases. These findings suggested that ROS signaling is an important part of lateral root development. It can be summarized that Prxs activities (regulated by UPB1) regulate emergence of lateral roots via ROS signaling, most probably by promoting transition from cellular proliferation to differentiation (as discussed in previous section; **Figure 2**).

ROS and Leaf Growth and Development

The complex coordination between cell proliferation and cell expansion decides the final size of a single organ or organism (Lu et al., 2014). Plant leaves are initiated by the proliferation of meristematic cells and then by cell expansion without additional division in a second phase (Beemster et al., 2005). An equilibrium between negative and positive regulators controls both phases by a large number of genetic pathways. For instance, TFs play a vital role (Townsley and Sinha, 2012). Of course, cell expansion is affected by changes in cell wall architecture and content (Bringmann et al., 2012; Rubio-Diaz et al., 2012). Such alterations may be due to Prxs that alter ROS levels in leaves (Passardi et al., 2004).

The Prxs particularly apoplastic, directly control stiffness of the cell wall, either by promoting or by restricting cellular extension (Tsukagoshi et al., 2010; Lee et al., 2013). In the first scenario, $O_2^{\bullet -}$ generated by cell wall peroxidases, promote

expansion by cleaving the cell wall polysaccharides and act as cell wall loosening agents (Müller et al., 2009). In contrast, H_2O_2 production in the cell wall promotes rigid cross-linking of cell wall components and results in growth restriction or making the cell wall stiffer (Passardi et al., 2004; Lu et al., 2014). The activity and expression of Prxs are suppressed by KUODA1 (KUA1), a MYB-like TF that was found to function as a positive regulator of cell expansion during leaf development by altering apoplastic ROS homeostasis in *A. thaliana* (Lu et al., 2014). The overexpression of KUA1 results in increased cell size with larger leaves without affecting the cell number (Lu et al., 2014). In contrast, the *kua1-1*mutant has smaller leaves than the wild type due to a decrease in cell size, while the cell number was again unaffected. Moreover, the *kua1* mutant shows elevated levels of H_2O_2 and increased activity of class III Prxs. The disturbance of KUA1 causes an increase in Prxs activity and results in smaller leaf cells. Therefore, expansion of the cell as well as the final size of the organ is controlled by ROS homeostasis as modulated by KUA1 (Lu et al., 2014). This positive regulation/promotion can be interlinked with change in apoplastic H_2O_2 levels and it is noteworthy that changes in H_2O_2 level can also affect the $O_2^{\bullet -}$ pool (Liszkay et al., 2003). Of note, in the case of KUA1, H_2O_2 mediated inhibitory effect of Prxs, seems to be the fundamental link for the regulation of leaf cell expansion. The equilibrium between $O_2^{\bullet -}$ and H_2O_2 regulates cell proliferation and differentiation zones that decide the size of an organ (as discussed in previous section). Lu et al. (2014) showed that suppression of Prxs expression by KUA1 increased leaf cell expansion, without causing the increase in leaf cell proliferation. Moreover, the size of the first cortical root cell was decreased by H_2O_2 treatments (Tsukagoshi et al., 2010), the root-localized Prxs are most probably essential to uphold low H_2O_2 levels to support root cell expansion. Contrary to this, Lu et al. (2014) also reported that inhibition of Prxs activity improves leaf size thereby suggesting that apoplastic Prxs mainly produce H_2O_2 that leads to cell wall crosslinking in leaves (Passardi et al., 2004). Therefore, the impact of Prxs specifically their effect on the H_2O_2 level seems to comprise greater opposing effects on leaf growth in plants.

CONCLUSION AND FUTURE DIRECTIVES

In spite of amazing development in our understanding of ROS biology, the exact nature of the ROS-signaling network largely remains mysterious. The present review is an attempt to adress the regulatory action of ROS in plant growth and development. Earlier these were considered to be toxic by-products only, but now they have been found to function as central players in complex signaling networks. We have tried here to uncover the beneficial role of ROS as signaling molecules. Undoubtedly, in the past decade, our understanding in the field of ROS signaling have significantly improved but miles we have to go. We are entering in an exciting era of ROS signaling in plants. Our stage is set and it's time to fit the different parts of the puzzle into its right place. Definitely, by uncovering novel biological roles of ROS, our understanding in the field of ROS signaling have become more than superficial, but many questions remain to be answered.

- For instance, is it possible to transport ROS from one subcellular site to another, and if possible, how do ROS signals journey within or across different cells?
- How are ROS-modulated gene networks linked with other signaling networks?
- Are they affected by changes in membrane potential?
- How does the effectiveness of ROS signals differ among sub-cellular compartments? How do NADPH oxidases participate in generating these signals?
- What are the concentrations of ROS in different sub-cellular compartments?
- Does each source contribute equally to the cellular ROS pool? Are specific ROS signatures induced by different stimuli?
- How do plant cells sense ROS?
- What are threshold levels of ROS at which they function as signaling molecules?

Answers to these questions are crucial for elucidating mechanisms of ROS signaling. Our current understating regarding the involvement of ROS in developmental processes largely depends on some discovered components of signaling pathways such as NOXs, OXI1, Ca^{2+} influx and efflux, and some TFs. Further studies are needed to properly explain the complex regulatory machinery that integrates ROS signals with components of signaling pathways for the regulation of growth and development of plants. ROS are associated with many biotic and abiotic responses, and interpreting ROS signaling is expected to have a noteworthy impact on biotechnology and agriculture, possibly leading to crop development by enhancing yield under suboptimal conditions. Future work will no doubt reveal novel signaling roles for ROS and their interaction with other signals, and hence future of ROS research is very promising.

AUTHOR CONTRIBUTIONS

VS and SP have conceptualized the review article. RS, SS, PP, RM, DT, VS, DC, and SP are being involved in writing this review article. RS and SS equally contributed in this review article.

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Abscisic Acid-Induced H_2O_2 Accumulation Enhances Antioxidant Capacity in Pumpkin-Grafted Cucumber Leaves under $Ca(NO_3)_2$ Stress

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With the aim to clarifying the role of the ABA/H_2O_2 signaling cascade in the regulating the antioxidant capacity of grafted cucumber plants in response to $Ca(NO_3)_2$ stress, we investigated the relationship between ABA-mediated H_2O_2 production and the activities of antioxidant enzymes in the leaves of pumpkin-grafted cucumber seedlings. The results showed that both ABA and H_2O_2 were detected in pumpkin-grafted cucumber seedlings in response to $Ca(NO_3)_2$ treatment within 0.5 h in the leaves and peaked at 3 and 6 h after $Ca(NO_3)_2$ treatment, respectively, compared to the levels under control conditions. The activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase (POD) in pumpkin-grafted cucumber leaves gradually increased over time and peaked at 12 h of $Ca(NO_3)_2$ stress. Furthermore, in the leaves of pumpkin-grafted cucumber seedlings, the H_2O_2 generation, the antioxidant enzyme activities and the expression of *SOD*, *POD* and *cAPX* were strongly blocked by an inhibitor of ABA under $Ca(NO_3)_2$ stress, but this effect was eliminated by the addition of exogenous ABA. Moreover, the activities and gene expressions of these antioxidant enzymes in pumpkin-grafted leaves were almost inhibited under $Ca(NO_3)_2$ stress by pretreatment with ROS scavengers. These results suggest that the pumpkin grafting-induced ABA accumulation mediated H_2O_2 generation, resulting in the induction of antioxidant defense systems in leaves exposed to $Ca(NO_3)_2$ stress in the ABA/H_2O_2 signaling pathway.

Keywords: abscisic acid, antioxidant enzymes, $Ca(NO_3)_2$ stress, cucumber, grafting, hydrogen peroxide

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INTRODUCTION

Greenhouse cultivation is the most common method of vegetable production worldwide. However, secondary soil salinization in greenhouse soil, which is primarily caused by over-irrigation, intensive farming, lack of rain, and excessive application of nitrogenous fertilizers, restricts the development and productivity of vegetables in China (Blanco and Folegatti, 2002; Liang et al., 2005;

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; CAT, catalase; DMTU, dimethylthiourea; DPI, diphenyleneiodonium chloride; POD, peroxidase; SOD, superoxide dismutase; Tiron, 1,2-dihydroxybenzene- 3,5-disulphonic acid.

Yu et al., 2005; He et al., 2007; Daliakopoulos et al., 2016). Several reports have indicated that the characteristics of greenhouse soil are different from those of coastal and inland saline soil. Its main cation is Ca^{2+}, and its main anion is NO_3^- , which account for 60 and 67–76% of the total cations and anions, respectively (Yuan et al., 2012; Xing et al., 2015). Excessive $Ca(NO_3)_2$ not only leads to osmotic stress, inhibits biological nitrogen fixation, and modifies microbial soil biodiversity but also causes the formation of reactive oxygen species (ROS) in plants. ROS can disorder the normal physiological metabolism and then inhibit plant growth and decrease crop yield (Sainju et al., 2001). However, plants have evolved adaptive protection mechanisms, such as antioxidant enzymes to protect themselves against the deleterious effects of ROS.

Cucumber (*Cucumis sativus* L.) is one of the most economically important vegetables worldwide, grown both in open fields and protected facilities. According to FAO statistics, the world cultivation area of cucumber was 2,115,457 hm^2 and the yield was 71,333,414 ton in 2013. In China, these two values were 1,166,690 hm^2 and 54,362,750 tons, respectively, making China the country with the largest production of cucumber (FAO, 2013). However, the yield of cucumber in China was only 46.6 tons·hm^{-2}, which was far lower than the highest yield (Netherlands, 666.7 ton·hm^{-2}), ranking China number 31 in the world. Because of their biological characteristics, cucumber plants are affected by many adverse environmental factors, of which the secondary salinization of greenhouse soil is a major problem, significantly decreasing the yield and quality of cucumber fruits. A common method of adapting plants to environmental stresses is by grafting commercial cultivars onto selected tolerant rootstocks (Lee and Oda, 2002). In horticultural crop production, grafting has already been used for more than 50 years in many regions of the world (Rivard and Louws, 2008). Vegetable seedlings grown under $Ca(NO_3)_2$ stress may possess higher contents of osmotic adjustment substances and higher activities of antioxidant enzymes if they are grafted with selected vigorous rootstocks and suffer less oxidative damage, contributing to a higher production and better quality of fruits (Zhang et al., 2008; Xing et al., 2015).

Abscisic acid has a wide range of physiological functions in higher plants, including regulating plant responses to various adverse environmental factors (Sah et al., 2016). ABA is considered a root-derived signaling molecule. It moves within plants, and its transport plays an important role in determining the endogenous hormone concentrations at the site of action (Seo and Koshiba, 2011). A number of studies suggest that ABA might be the chemical substance responsible for root-to-shoot signaling, especially under abiotic stress conditions (Sah et al., 2016). An increasing body of evidence indicates that one mode of ABA action is related to oxidative stress in plant cells. It is well known that ABA can increase the generation of H_2O_2 (Kwak et al., 2003; Laloi et al., 2004); cause the gene expression of superoxide dismutase (SOD), CAT, and APX, increase the activities of these antioxidant enzymes in plant tissues; and enhance the stress resistance of plants (Guan et al., 2000; Jiang and Zhang, 2003; Park et al., 2004; Saxena et al., 2016).

However, little information exists about whether the increased enzyme activities of antioxidants induced by ABA in an H_2O_2-dependent way would occur in pumpkin-grafted cucumber seedlings and lead to better plant performance than that in self-grafted cucumber seedlings under $Ca(NO_3)_2$ stress. In this study, the ABA and H_2O_2 contents and antioxidant enzyme activities in the leaves of pumpkin-grafted and self-grafted cucumber seedlings under $Ca(NO_3)_2$ stress were examined. In addition, this study investigated whether the increased activities of antioxidant enzymes and their encoded gene (*SOD, POD,* and *cAPX*) expression in pumpkin-grafted cucumber leaves under $Ca(NO_3)_2$ stress are induced by ABA and whether H_2O_2 is involved in this induction. The mechanism by which the pumpkin rootstock enhances antioxidant defense for the $Ca(NO_3)_2$ stress tolerance of cucumber seedlings is also discussed.

MATERIALS AND METHODS

Plant Material and Treatments

Cucumber cultivar (*Cucumis sativus* L. 'Jinyou No. 3', obtained from Tianjin Kerun Research Institution) was used as the scion. A salt-tolerant pumpkin 'Qingzhen 1' (*Cucurbita maxima* × *Cucurbita moschata*, obtained from Qingdao Agriculture Academy of Science) was selected as the rootstock (Xing et al., 2015). Cucumber and pumpkin seeds were surface sterilized with 1% (v/v) sodium hypochlorite, washed thoroughly with distilled water, and then sown in plastic salvers (41 cm × 41 cm × 5 cm) containing quartz sand and incubated in a greenhouse at Nanjing Agriculture University, China. The average day/night temperatures in the greenhouse were at 25–30°C/15–18°C, and the relative humidity was 60–75%. When the scion's cotyledons were fully expanded and the rootstock's second true leaves were in the development stage, the insert grafting procedure was performed as described by Lee and Oda (2002). Self-grafted plants were included as the controls. Uniformly sized pumpkin-grafted seedlings were grown hydroponically in plastic containers filled with half-strength Hoagland's solution (pH 6.5 ± 0.1, EC 2.0–2.2 dS·m^{-1}) for the next experiments. The solution was replaced every 3 days and continuously aerated with an air pump at an interval of 20 min to keep the dissolved oxygen level at 8.0 ± 0.2 mg·L^{-1}.

After the full development of the third true leaves, the seedlings were treated as follows: 80 mM $Ca(NO_3)_2$ was added to the solution for the salt stress treatment. Different exogenous substances such as 10 μM ABA, 1 mM sodium tungstate (T, an ABA synthesis inhibitor), 10 mM 1,2-dihydroxybenzene- 3,5-disulfonic acid (Tiron, a specific $O_2^{\bullet-}$ scavenger), 100 μM DPI (a specific NADPH oxidase inhibitor) and 5 mM DMTU (a specific H_2O_2 scavenger) were used in various treatments for 8 h before salt stress treatment. All of the treatments were as follows:

(a) S-G, self-grafted cucumber seedlings grown in Hoagland's solution;
(b) S-GN, self-grafted cucumber seedlings grown in Hoagland's solution with 80 mM $Ca(NO_3)_2$;

(c) P-G, pumpkin-grafted cucumber seedlings grown in Hoagland's solution;
(d) P-GN, pumpkin-grafted cucumber seedlings grown in Hoagland's solution with 80 mM $Ca(NO_3)_2$;
(e) P-GN + T, pumpkin-grafted cucumber seedlings pretreated with 1 mM sodium tungstate grown in Hoagland's solution with 80 mM $Ca(NO_3)_2$;
(f) P-GN + T + ABA, pumpkin-grafted cucumber seedlings pretreated with 1 mM sodium tungstate and 10 μM ABA grown in Hoagland's solution with 80 mM $Ca(NO_3)_2$;
(g) P-GN + DPI, pumpkin-grafted cucumber seedlings pretreated with 100 μM DPI grown in Hoagland's solution with 80 mM $Ca(NO_3)_2$;
(h) P-GN + Tiron, pumpkin-grafted cucumber seedlings pretreated with 10 mM Tiron grown in Hoagland's solution with 80 mM $Ca(NO_3)_2$;
(i) P-GN + DMTU, pumpkin-grafted cucumber seedlings pretreated with 5 mM DMTU grown in Hoagland's solution with 80 mM $Ca(NO_3)_2$.

The experiments were arranged in a randomized complete block design with three replicates. Each treatment included three containers with 36 seedlings. The third fully expanded leaves (from the top) were sampled at key time points as indicated below and immediately frozen in liquid nitrogen.

ABA Assay

Fresh leaves were homogenized in an extraction solution containing 80% methanol, 0.05% citric acid and 0.45 mM butylated hydroxytoluene and were then centrifuged at 8,000 × g for 10 min. The samples were dried, and the radioactivity in the pellet was quantified. ABA was assayed using radioimmunoassay as described by Verslues and Bray (2006).

Measurement of the H_2O_2 Level

The H_2O_2 content was measured according to the method described by Brennan and Frenkel (1977). The absorbance values via OD at 415 nm were calibrated to a standard graph generated with known concentrations of H_2O_2.

Measurement of the Antioxidant Enzyme Activity

For extract of antioxidant enzymes, fresh leaves were homogenized with 1.6 mL of 50 mM phosphate buffer (pH 7.8) containing 1 mM EDTA and 2% PVP. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C, and the resulting supernatant was used to assay enzyme activity as follows: the SOD activity was assayed by monitoring the inhibition of the photochemical reduction of NBT following the method of Giannopolitis and Ries (1997). One unit of SOD activity was defined as the amount of enzyme that was required to cause a 50% inhibition of the reduction of NBT as monitored at 560 nm.

The POD activity was measured according to the method of Rao et al. (1996) with slight modification. For this, 5 mL of the extracted enzyme was mixed with 3 mL of the reaction mixture containing 50 mM PBS (pH 7.0) and 20 mM guaiacol. After pre-incubation at 25°C for 5 min, 6 mM H_2O_2 was added to initiate the reaction. Changes in the absorbance at 470 nm within 2 min were recorded to calculate POD activity. One unit of POD activity was expressed as $U.g^{-1}$ FW.

A modified method from Aebi (1974) was used to assay the CAT activity. For this, 100 mL of the extraction was added to 3 mL 50 mM PBS buffer (pH 7.0). After incubation, the reaction was started by the addition of 6 mM H_2O_2. The CAT activity was expressed as $U.g^{-1}$ FW.

The APX activity was performed as described by Pinhero et al. (1997). The assay was carried out in a reaction mixture consisting of 50 mM PBS (pH7.0), 0.5 mM AsA, 3 mM H_2O_2 and 100 mL of the extraction. One unit of APX activity was defined as an absorbance change of 0.1 unit min^{-1}, and the APX activity was expressed as $U.g^{-1}$ FW.

RNA Isolation and Quantitative Real-Time (qRT-PCR) Analysis

Total RNA was extracted from leaves as described in the TRI reagent protocol (Takara Bio Inc). Primers were designed according to cucumber databases[1]. and NCBI. Gene specific primers used for real-time quantitative PCR are provided in the following primers: *SOD*: forward CCTAAACTCTCGT GAATGA and reverse CAGCAGACAAGTATGGATA; *POD*: forward TTGTAATAATGGCGGCTT and reverse GTGTCATA GAAGGTGGAG; *cAPX*: forward TGCTTTCATCACCATCAA and reverse TGTTATGTTCTTGTCTTCCT. *Actin*: forward CCACCAATCTTGTACACATCC and reverse AGACCACCAA GTACTACTGCAC. qRT-PCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using a SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara). The PCR reactions were carried out in triplicate and the thermocycler conditions, 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and a final extension of 30 s at 60°C. Relative expression was calculated according to the $2^{-\Delta\Delta CT}$ method, the relative gene expression level was normalized against *actin* (the internal standard gene).

Statistical Analysis

All data were statistically analyzed with SAS 13.0 software (SAS Institute, Inc., Cary, NC, USA) using Duncan's multiple range test at the $P < 0.05$ level of significance.

RESULTS

Effects of $Ca(NO_3)_2$ Stress on the Contents of ABA and H_2O_2 in the Leaves of Pumpkin-Grafted and Self-Grafted Cucumber Seedlings

Compared to their corresponding controls, the ABA content in the leaves of pumpkin-grafted and self-grafted seedlings significantly increased under 80 mM $Ca(NO_3)_2$ stress (**Figure 1**), whereas the ABA level in the rootstock-grafted leaves was higher than that of the self-grafted leaves under $Ca(NO_3)_2$ stress.

[1]http://cucumber.genomics.org.cn

The ABA content in the leaves of pumpkin-grafted cucumber seedlings increased after 0.5 h of $Ca(NO_3)_2$ stress and peaked at 3 h. However, the ABA content in the leaves of the self-grafted cucumber seedlings did not significant changes after the entire duration of $Ca(NO_3)_2$ stress.

The H_2O_2 content in the leaves of pumpkin-grafted and self-grafted cucumber seedlings increased by $Ca(NO_3)_2$ stress compared to the corresponding controls (**Figure 2**). The H_2O_2 content increased after 0.5 h of $Ca(NO_3)_2$ stress and peaked at 6 h, after which it rapidly decreased in the leaves of pumpkin-grafted and self-grafted cucumber seedlings. However, the pumpkin-grafted seedlings had a higher H_2O_2 level than that of the self-grafted seedlings leaves during the treatment of $Ca(NO_3)_2$ stress. Under non-saline conditions, there were no significant differences in the H_2O_2 level between self-grafted seedlings and pumpkin-grafted cucumber leaves.

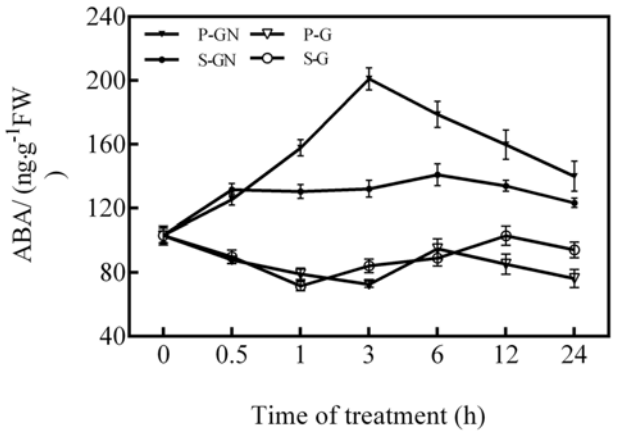

FIGURE 1 | Time course of changes in the ABA content in the leaves of self-grafted and pumpkin-grafted cucumber seedlings exposed to 80 mM $Ca(NO_3)_2$. Each value is the mean ± SE of six independent experiments ($n = 6$). P-G, pumpkin-grafted cucumber seedlings grown in Hoagland's solution; P-GN, pumpkin-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$; S-G, self-grafted cucumber seedlings grown in Hoagland's solution; S-GN, self-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$.

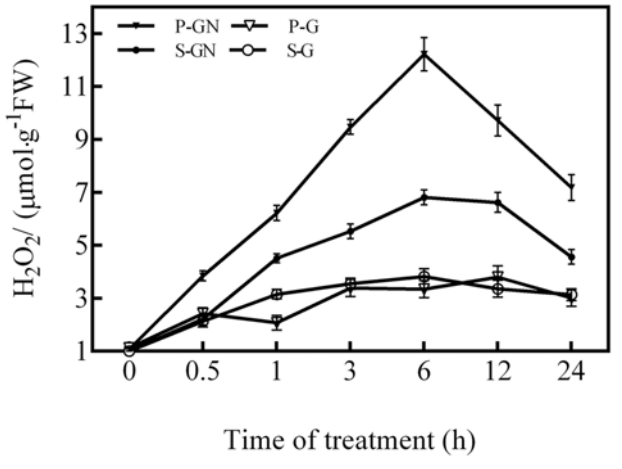

FIGURE 2 | Time course of changes in the H_2O_2 content in the leaves of self-grafted and pumpkin-grafted cucumber seedlings exposed to 80 mM $Ca(NO_3)_2$. Each value is the mean ± SE of six independent experiments ($n = 6$). P-G, pumpkin-grafted cucumber seedlings grown in Hoagland's solution; P-GN, pumpkin-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$; S-G, self-grafted cucumber seedlings grown in Hoagland's solution; S-GN, self-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$.

Effects of $Ca(NO_3)_2$ Stress on the Activities of Antioxidant Enzymes in the Leaves of Pumpkin-Grafted and Self-Grafted Cucumber Seedlings

Treatment with $Ca(NO_3)_2$ stress led to similar changes of in the antioxidant enzyme activities in the leaves of pumpkin-grafted and self-grafted cucumber seedlings, but the changes in the activities of antioxidant enzymes in the rootstock-grafted leaves were more significant and the range was much wider than those of the self-grafted seedlings (**Figure 3**). Compared to their corresponding controls, the activities of SOD, POD and CAT increased in the leaves of pumpkin-grafted and self-grafted cucumber seedlings under $Ca(NO_3)_2$ stress. Moreover, these values of the pumpkin-grafted seedlings were increasingly higher than those of the self-grafted seedlings under $Ca(NO_3)_2$ stress. The activities of SOD, POD and APX in the pumpkin-grafted seedlings leaves increased after 6 h of $Ca(NO_3)_2$ stress and peaked at 12 h, after which it gradually decreased. The activity of CAT in the leaves increased and peaked at 12 h, then decreased at 24 h, and then increased until 48 h in pumpkin-grafted and self-grafted cucumber seedlings under $Ca(NO_3)_2$ stress (Gao et al., 2015). Under non-saline conditions, there was no significant difference in the activities of antioxidant enzymes between pumpkin-grafted and self-grafted seedlings leaves, except for the APX activity at 12 and 36 h.

ABA-Mediated Accumulation of H_2O_2 in the Leaves of Pumpkin-Grafted Seedlings under $Ca(NO_3)_2$ Stress

Sodium tungstate (T), an ABA synthesis inhibitor, and exogenous ABA were used to determine whether ABA is involved in inducing H_2O_2 accumulation in pumpkin-grafted cucumber seedlings under $Ca(NO_3)_2$ stress. $Ca(NO_3)_2$ stress significantly increased the H_2O_2 content of the pumpkin-grafted seedlings leaves compared to that of the control, but pretreatment with 5 mM sodium tungstate remarkably inhibited the increased H_2O_2 induced by $Ca(NO_3)_2$ stress. However, the application of exogenous 100 μM ABA to $Ca(NO_3)_2$ stress alleviated the inhibition of the H_2O_2 content in the leaves of the pumpkin-grafted seedlings with sodium tungstate (**Figure 4**).

ABA Involved in Enhancing Antioxidant Capacity in Pumpkin-Grafted Seedlings Leaves under $Ca(NO_3)_2$ Stress

Abscisic acid synthesis inhibitor T was used to determine the effects of ABA on the antioxidant defense of pumpkin-grafted cucumber seedlings under $Ca(NO_3)_2$ stress. According to the

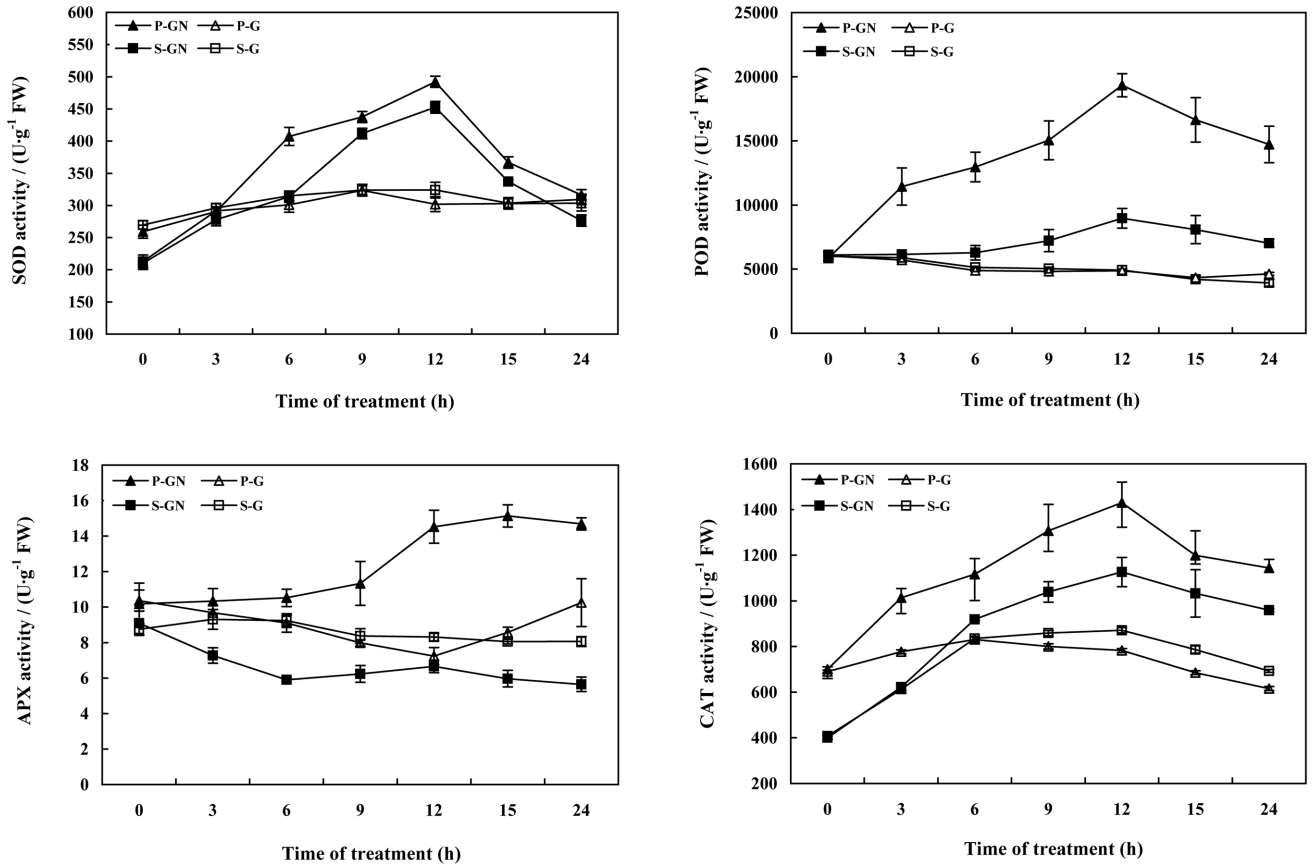

FIGURE 3 | Time course of changes in the activities of SOD, POD, CAT, and APX in the leaves of self-grafted and pumpkin-grafted cucumber seedlings exposed to 80 mM $Ca(NO_3)_2$. Each value is the mean ± SE of six independent experiments ($n = 6$). P-G, pumpkin-grafted cucumber seedlings grown in Hoagland's solution; P-GN, pumpkin-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$; S-G, self-grafted cucumber seedlings grown in Hoagland's solution; S-GN, self-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$.

results in **Figure 3**, an irregular change in CAT activity was induced by $Ca(NO_3)_2$ stress; thus, we only studied the other three enzymes. Pretreatment with sodium tungstate significantly inhibited the increase in the SOD, POD and APX activities in the leaves of the pumpkin-grafted seedlings under $Ca(NO_3)_2$ stress (**Figure 5**). However, exogenous ABA in addition to $Ca(NO_3)_2$ stress alleviated the inhibition of the SOD, POD and APX activities of the pumpkin-grafted seedlings in the presence of sodium tungstate. We analyzed the expression profiles of three transcripts that encoded SOD, POD and APX using real-time quantitative RT-PCR to evaluate the correlation between these enzyme activities and their encoded gene expression after 12 h of $Ca(NO_3)_2$ stress (**Figure 6**). The *SOD*, *POD,* and *cAPX* expression patterns were similar to their enzyme activities under different treatment conditions.

H_2O_2 Accumulation Induced Antioxidant Defense in Pumpkin-Grafted Seedlings Leaves under $Ca(NO_3)_2$ Stress

Reactive oxygen species are key signaling molecules for stress tolerance in plants. We thus examined the effects of pretreatment with ROS manipulators (DPI, Tiron and DMTU) on the gene expression and activities of antioxidant enzymes in pumpkin-grafted seedling leaves. $Ca(NO_3)_2$ stress induced a significant increase in the activities of SOD, POD and APX and their corresponding gene expression in pumpkin-grafted cucumber leaves, and these positive effects were completely prevented by pretreatment with DPI, Tiron and DMTU (**Figures 7** and **8**). These results indicate that ABA is involved in H_2O_2-accumulation-mediated antioxidant defense in pumpkin-grafted plants and in the subsequently improved $Ca(NO_3)_2$ tolerance of cucumber seedlings.

DISCUSSION

It is well known that grafting with stress-tolerant rootstock can enhance plant tolerance to stress. In our previous studies, the $Ca(NO_3)_2$ stress tolerance of cucumber seedlings could be enhanced by grafting with pumpkin from different physiological aspects, including osmotic adjustment ability, nitrogen metabolism, soluble protein expression and antioxidant defense (Wang et al., 2012; Xing et al., 2015), but little

information is available about the roles of the ABA and H_2O_2 signaling pathways in relieving $Ca(NO_3)_2$ stress. In this study, we present evidence that grafting-induced ABA accumulation in cucumber leaves triggered H_2O_2 production, thus enhancing activities of antioxidant enzymes, the expression of their encoding gene and the subsequent salt tolerance in response to $Ca(NO_3)_2$ stress.

Abscisic acid is the most important phytohormone that has multiple functions in the developmental processes of plants and enhances plants tolerance to various stresses including salinity, drought, and low temperature (Giraudat et al., 1994; Sah et al., 2016). In this study, the ABA content greatly increased in both self-grafted and rootstock-grafted cucumber leaves during the $Ca(NO_3)_2$ treatment (**Figure 1**). The increased ABA levels by grafting under stressed conditions probably resulted not only from the increased catabolism via the mevalonic acid-independent pathway but also from the translocation of other tissues or organs (Roychoudhury et al., 2013). Moreover, we also observed that the increased ABA content in the leaves of the rootstock-grafted plants was higher than that of the self-grafted plants under $Ca(NO_3)_2$ stress. These results may indicate that grafting cucumber seedlings with pumpkin rootstock exhibited a strong ability to resist $Ca(NO_3)_2$ stress. It has also been shown that increased endogenous ABA activates a complex signaling network leading to cellular responses to stresses (Ng et al., 2014). Several studies have shown that ABA accumulation induced by abiotic stresses could have a physiological effect on ROS production (Pei et al., 2000; Zhang et al., 2006).

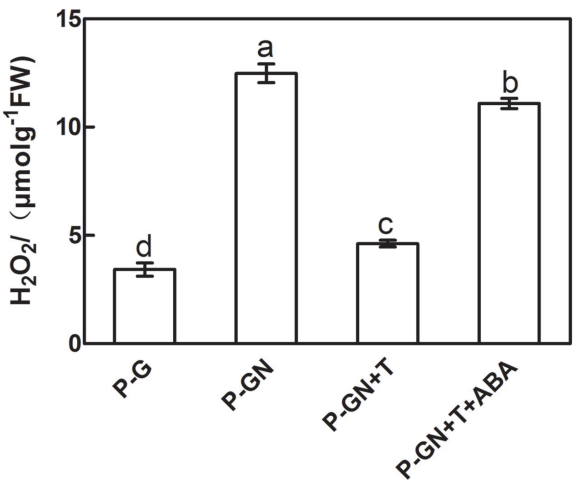

FIGURE 4 | Effects of pretreatment with the ABA inhibitor sodium tungstate (T) and exogenous ABA on the content of H_2O_2 in the leaves of pumpkin-grafted cucumber seedlings exposed to 80 mM $Ca(NO_3)_2$ stress. The pumpkin-grafted cucumber seedlings were pretreated with 5 mM T (P-GN + T) and 100 μM exogenous ABA (P-GN + T + ABA) for 8 and 12 h, respectively, and then exposed to 80 mM $Ca(NO_3)_2$ for 12 h. Each histogram represents a mean value of three independent experiments, and the vertical bars indicate SE ($n = 6$). Different letters indicate significant differences at $P < 0.05$, according to Duncan's multiple range tests. P-G, pumpkin-grafted cucumber seedlings grown in Hoagland's solution; P-GN, pumpkin-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$.

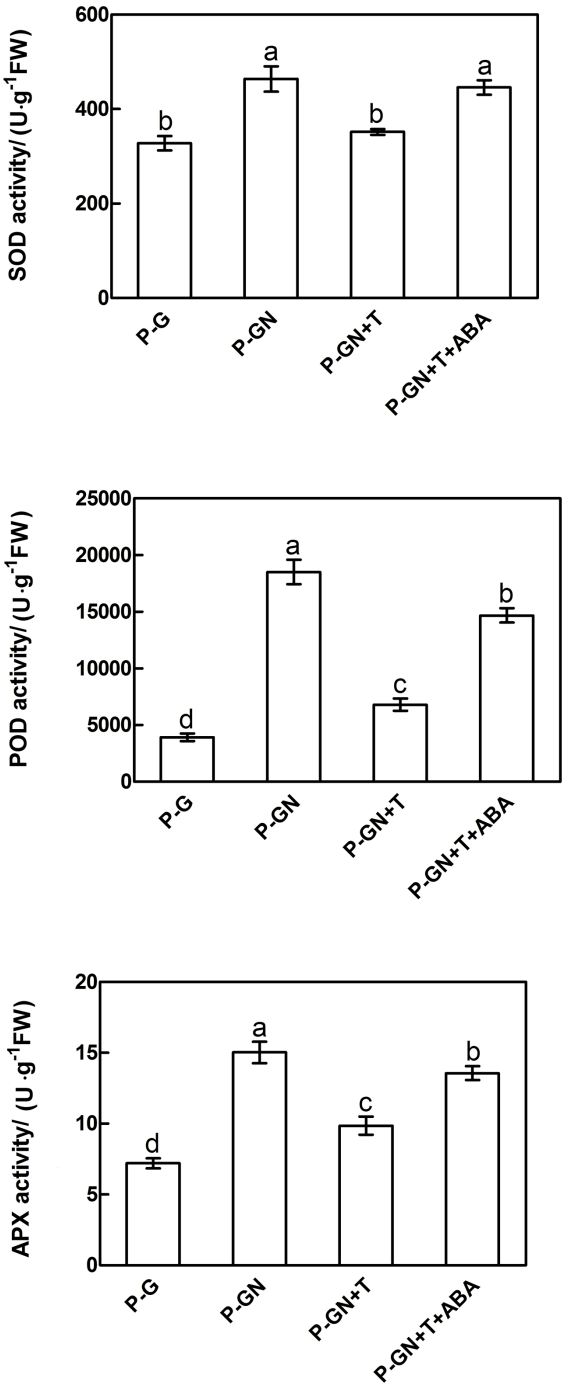

FIGURE 5 | Effects of pretreatment with the ABA inhibitor sodium tungstate (T) and exogenous ABA on the activities of SOD, POD and APX in the leaves of pumpkin-grafted cucumber seedlings exposed to mM $Ca(NO_3)_2$. The pumpkin-grafted cucumber seedlings were pretreated with 5 mM T (P-GN + T) and 100 μM exogenous ABA (P-GN + T + ABA) for 8 and 12 h, respectively, and then exposed to 80 mM $Ca(NO_3)_2$ for 12 h. Each histogram represents the mean value of three independent experiments, and the vertical bars indicate SE ($n = 6$). Different letters indicate significant differences at $P < 0.05$, according to Duncan's multiple range tests. P-G, pumpkin-grafted cucumber seedlings grown in Hoagland's solution; P-GN, pumpkin-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$.

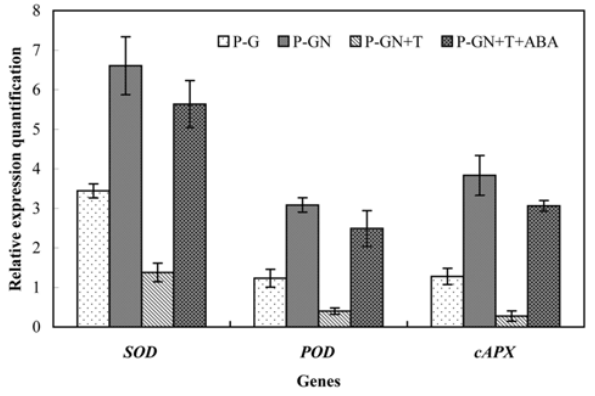

FIGURE 6 | Effects of pretreatment with the ABA inhibitor sodium tungstate (T) and exogenous ABA on the expression of *SOD*, *POD,* and *APX* in the leaves of pumpkin-grafted cucumber seedlings exposed to 80 mM $Ca(NO_3)_2$. The pumpkin-grafted cucumber seedlings were pretreated with 5 mM T (P-GN + T) and 100 μM exogenous ABA (P-GN + T + ABA) for 8 and 12 h, respectively, and then exposed to 80 mM $Ca(NO_3)_2$ for 12 h. Each histogram represents the mean value of three independent experiments, and the vertical bars indicate SE ($n = 3$). P-G, pumpkin-grafted cucumber seedlings grown in Hoagland's solution; P-GN, pumpkin-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$.

As secondary messengers, ROS mediate variety of physiological functions and defense responses against abiotic stresses in plants. These functions include the regulation of seed germination (Roach et al., 2010), root development (Foreman et al., 2003), photosynthesis (Foyer et al., 2012), senescence (Gao et al., 2016), and adaptive responses to abiotic stresses (Jiang and Zhang, 2002a,b; Saxena et al., 2016). H_2O_2, a type of ROS, generates rapidly in plants in response to stress conditions (Saxena et al., 2016). However, it is not clear whether there exist similar responses in grafted plants, especially in plants exposed to $Ca(NO_3)_2$ stress. In this study, our results showed that $Ca(NO_3)_2$ stress increased the ABA and H_2O_2 contents and the antioxidant defense of pumpkin-grafted cucumber leaves. It is essential for plants to maintain the interaction between ABA and H_2O_2 to avoid any oxidative stress induced by adverse environmental factors (Saxena et al., 2016). As the time course of the production of ABA (**Figure 1**) and H_2O_2 (**Figure 2**) showed, the peak time of ABA production (3 h) preceded that of H_2O_2 production (6 h); then, at 12 h of $Ca(NO_3)_2$ treatment, the activities of the antioxidant enzymes SOD, POD and APX peaked. It was hypothesized that the antioxidant defense induced by $Ca(NO_3)_2$ stress in the leaves of pumpkin-grafted cucumber plants was initiated by ABA and involved H_2O_2.

To test our hypothesis, subsequent experiments with different inhibitors and scavengers, such as the ABA inhibitor sodium tungstate (T) and the H_2O_2 scavenger DMTU, were performed. Our results showed that the increased H_2O_2 content under $Ca(NO_3)_2$ stress was blocked in the pumpkin-grafted cucumber leaves that were pretreated with T (**Figure 4**). However, the decrease in the H_2O_2 level of the pumpkin-grafted plants with T recovered by the application of exogenous ABA. These

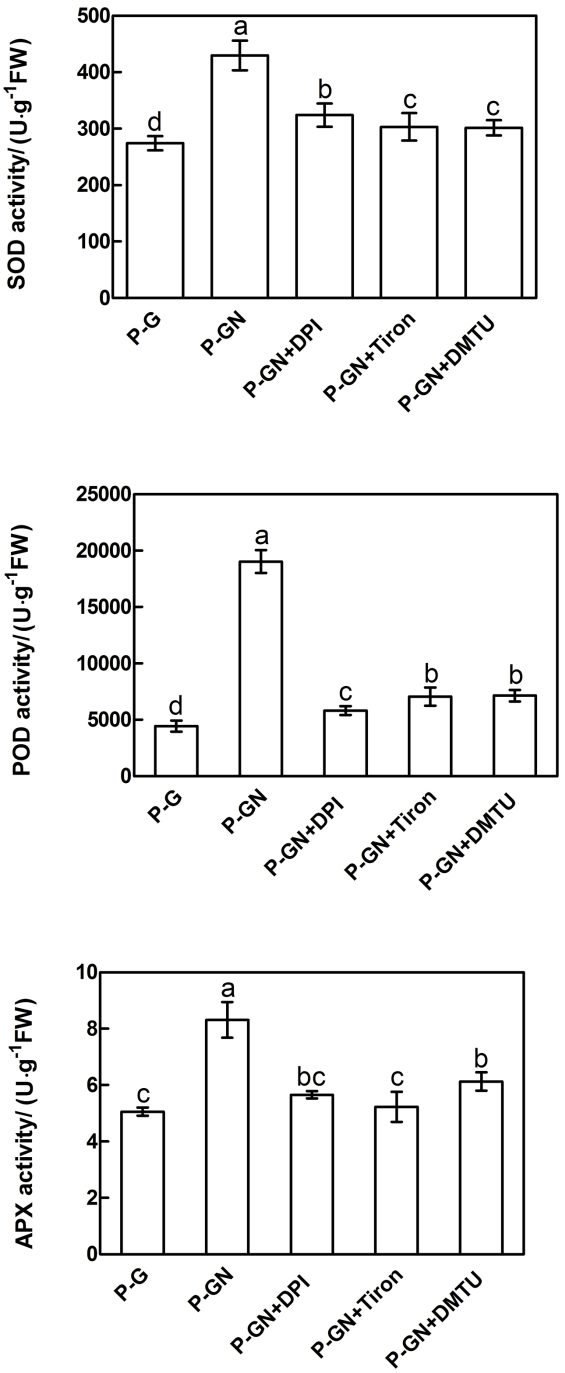

FIGURE 7 | Effects of pretreatment with the NADPH oxidase inhibitor diphenyleneiodonium (DPI), the O_2^- scavenger Tiron and the H_2O_2 scavenger DMTU on the activities of SOD, POD, and APX in the leaves of pumpkin-grafted cucumber seedlings exposed to 80 mM $Ca(NO_3)_2$. The pumpkin-grafted cucumber seedlings were pretreated with 100 μM DPI (P-GN + DPI), 10 mM Tiron (P-GN + Tiron), and 5 mM DMTU (P-GN + DMTU) for 8 h. Each histogram represents the mean value of three independent experiments, and the vertical bars indicate SE ($n = 6$). Different letters indicate significant differences at $P < 0.05$, according to Duncan's multiple range tests. P-G, pumpkin-grafted cucumber seedlings grown in Hoagland's solution; P-GN, pumpkin-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$.

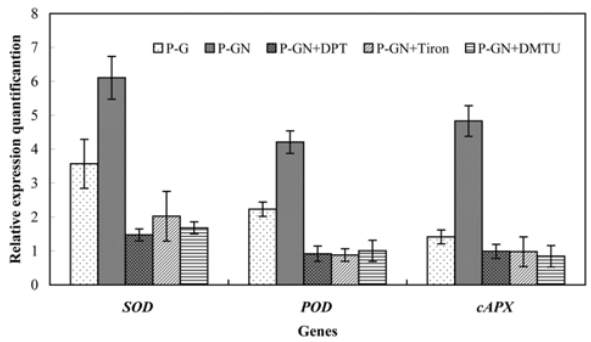

FIGURE 8 | Effects of pretreatment with the NADPH oxidase inhibitor diphenyleneiodonium (DPI), the O_2^- scavenger Tiron and the H_2O_2 scavenger DMTU on the expression of the *SOD*, *POD,* and *cAPX* in pumpkin-grafted cucumber seedlings leaves exposed to 80 mM $Ca(NO_3)_2$. The pumpkin-grafted cucumber seedlings were pretreated with 100 μM DPI (P-GN + DPI), 10 mM Tiron (P-GN + Tiron), and 5 mM DMTU (P-GN + DMTU) for 8 h. Each histogram represents the mean value of three independent experiments, and the vertical bars indicate SE ($n = 3$). P-G, pumpkin-grafted cucumber seedlings grown in Hoagland's solution; P-GN, pumpkin-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$.

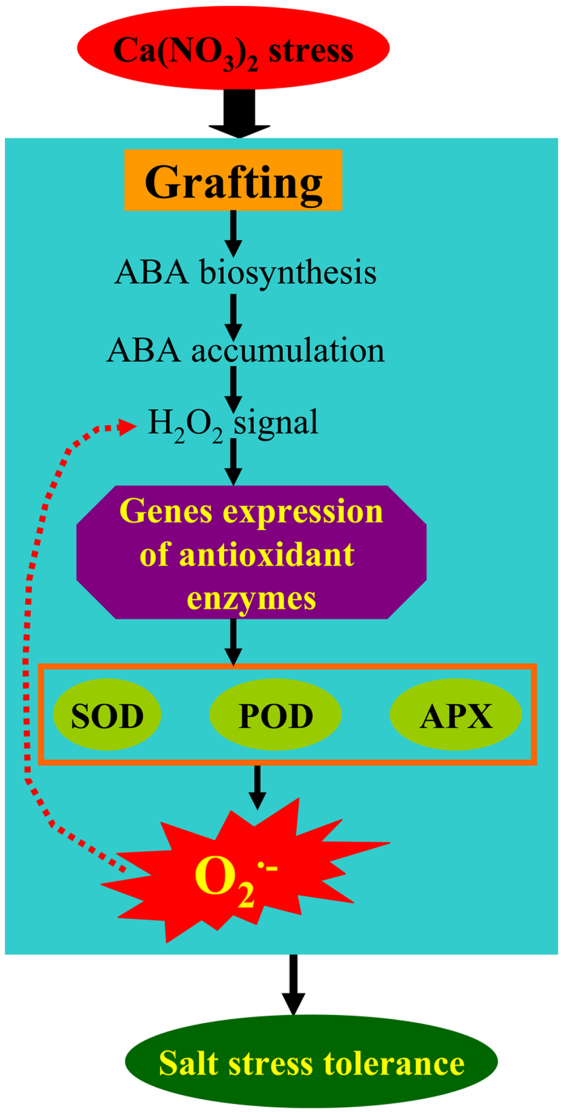

FIGURE 9 | A simplified metabolic scheme and signal transduction pathway for ABA and H_2O_2 in the leaves of pumpkin-grafted cucumber seedlings exposed to 80 mM $Ca(NO_3)_2$. The model is based on the results presented here. The red arrow (dotted line) indicates possible metabolic pathways in our experiment.

results suggest a crosslink between ABA and H_2O_2 signaling pathways. Previous studies have also clearly demonstrated that the increased H_2O_2 levels depend on the activation of ABA in pumpkin-grafted cucumber leaves under $Ca(NO_3)_2$ stress. Similar result was observed by Guan et al. (2000), who showed that endogenous H_2O_2 level significantly increased under high concentrations of ABA conditions. In addition, the antioxidant defense of leaves induced by $Ca(NO_3)_2$ treatment was significantly inhibited in pumpkin-grafted cucumber plants that were pretreated with T (**Figure 5**). T blocks the formation of ABA from abscisic aldehyde by impairing abscisic aldehyde oxidase (Hansen and Grossmann, 2000). These results suggest that ABA was required for the increased $Ca(NO_3)_2$ stress-induced H_2O_2 production and antioxidant defense in grafted cucumber plants.

It has been shown that ABA-induced ROS production increases the activities of SOD, CAT, APX and GR in maize (Jiang and Zhang, 2002a,b). Recent studies have determined that ABA and H_2O_2 induced activation of antioxidant enzymes by using transgenic tobacco plants in combination with their inhibitors or scavengers (Lu et al., 2014). In the present study, pretreatment with several ROS manipulators, such as the NADPH oxidase inhibitor DPI, the $O_2^{\bullet-}$ scavenger Tiron and the H_2O_2 scavenger DMTU, almost completely depressed ABA-induced antioxidant defense in the leaves of pumpkin-grafted cucumber plants under $Ca(NO_3)_2$ stress (**Figure 6**). In the leaves of maize seedlings, NADPH oxidase is involved in ABA-induced ROS production (Jiang and Zhang, 2002b). This may induce oxidative damage to plant cells, resulting in disrupted metabolic function and destroyed cellular integrity (Ozden et al., 2009). H_2O_2 generation induced by NADPH oxidase might be as a reaction cascade that triggers the antioxidant enzyme activities in *Arabidopsis thaliana*, thereby mitigating the salt stress-induced oxidative damage (Rejeb et al., 2015). According to the review by Forman (2007), H_2O_2 can be toxic to plants but can also be an important stress signal. H_2O_2 can be synthesized in response to exogenous ABA. H_2O_2 mediates, at least in part, ABA responses, including defense mechanisms, stomatal closure and gene expression (Guan et al., 2000; Pei et al., 2000; Saxena et al., 2016). Desikan et al. (2001) provided further evidence of H_2O_2 as a central signaling mediator of the abiotic stress response in plants by using cDNA microarray technology. Their studies showed that oxidative stress induced the expression of some genes, such as *SLN1-SSK1* (a gene encoding a potential hybrid His kinase) and that *MAPKKs* (MAPK kinases) are up-regulated by H_2O_2. This evidence suggests that H_2O_2 was involved in the ABA-induced

antioxidant defense in the leaves of pumpkin-grafted seedlings, thus enhancing cucumber tolerance in responses to $Ca(NO_3)_2$ stress.

CONCLUSION

In the present study, our results indicate that the ABA responses of pumpkin-grafted and self-grafted cucumber leaves were differently induced by 80 mM $Ca(NO_3)_2$ stress. The accumulation of ABA was involved in the rapid accumulation of H_2O_2, and the accumulation of H_2O_2 induced the activities of SOD, POD and APX and the expression of their encoded genes in pumpkin-grafted cucumber leaves (**Figure 9**). The higher capacity of antioxidant defense in the pumpkin-grafted cucumber plants induced by the ABA signaling pathways presented in our studies may be part of the reason for the better performance of these plants than that of self-grafted cucumber plants under $Ca(NO_3)_2$ stress (**Supplementary Figure S1**). The specific mechanism of ABA-H_2O_2 signaling requires further investigation to obtain more insight into the root-shoot signaling in rootstock-grafted cucumber plants.

AUTHOR CONTRIBUTIONS

SS wrote the main manuscript text. PG, LL and YY prepared all figures and modified this manuscript until submitted. JS performed the experiments. SG designed the research and proposed the research proceeding. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01489

FIGURE S1| Phenotypic response of self-grafted and pumpkin-grafted cucumber seedlings exposed to 80 mM $Ca(NO_3)_2$ for 7 days. P-G, pumpkin-grafted cucumber seedlings grown in Hoagland's solution; P-GN, pumpkin-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$; S-G, self-grafted cucumber seedlings grown in Hoagland's solution; S-GN, self-grafted cucumber seedlings with 80 mM $Ca(NO_3)_2$.

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ROS-Mediated Inhibition of *S*-nitrosoglutathione Reductase Contributes to the Activation of Anti-oxidative Mechanisms

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Nitric oxide (NO) has emerged as a signaling molecule in plants being involved in diverse physiological processes like germination, root growth, stomata closing and response to biotic and abiotic stress. *S*-nitrosoglutathione (GSNO) as a biological NO donor has a very important function in NO signaling since it can transfer its NO moiety to other proteins (*trans*-nitrosylation). Such *trans*-nitrosylation reactions are equilibrium reactions and depend on GSNO level. The breakdown of GSNO and thus the level of *S*-nitrosylated proteins are regulated by GSNO-reductase (GSNOR). In this way, this enzyme controls *S*-nitrosothiol levels and regulates NO signaling. Here we report that *Arabidopsis thaliana* GSNOR activity is reversibly inhibited by H_2O_2 *in vitro* and by paraquat-induced oxidative stress *in vivo*. Light scattering analyses of reduced and oxidized recombinant GSNOR demonstrated that GSNOR proteins form dimers under both reducing and oxidizing conditions. Moreover, mass spectrometric analyses revealed that H_2O_2-treatment increased the amount of oxidative modifications on Zn^{2+}-coordinating Cys47 and Cys177. Inhibition of GSNOR results in enhanced levels of *S*-nitrosothiols followed by accumulation of glutathione. Moreover, transcript levels of redox-regulated genes and activities of glutathione-dependent enzymes are increased in *gsnor-ko* plants, which may contribute to the enhanced resistance against oxidative stress. In sum, our results demonstrate that reactive oxygen species (ROS)-dependent inhibition of GSNOR is playing an important role in activation of anti-oxidative mechanisms to damping oxidative damage and imply a direct crosstalk between ROS- and NO-signaling.

Keywords: nitric oxide, *S*-nitrosoglutathione reductase, *S*-nitrosothiols, reactive oxygen species, oxidative stress, hydrogen peroxide, paraquat, *Arabidopsis thaliana*

INTRODUCTION

Plants are continuously facing to the changing environment that affects plant growth and productivity. To deal with multiple stress conditions, plants have developed adaptive responses. Nitric oxide (NO) as a signaling molecule plays a crucial role in these responses acting alone or together with reactive oxygen species (ROS) to regulate hormonal signaling pathways, gene expression changes or protein activities. The regulatory role of NO has been demonstrated in response to abiotic and biotic stresses as well as in plant developmentally processes throughout the entire plant life (Corpas et al., 2011; Yu et al., 2014; Simontacchi et al., 2015). NO can influence protein activity, translocation and protein function by posttranslational modifications. The predominant way of NO action is the reversible *S*-nitrosylation, a covalent attachment of NO to cysteine thiols. Further modifications are the nitrosylation of metal center of metalloproteins and the irreversible nitration of protein tyrosine residues (Astier and Lindermayr, 2012; Kovacs and Lindermayr, 2013). As a free radical, NO has a very short lifetime that restrict their effect to the local microenvironment. However, *S*-nitrosylated glutathione (*S*-nitrosoglutathione GSNO) is a quite stable NO reservoir and NO transport form. GSNO can *trans*-nitrosylate proteins regulating their activity/function (Lindermayr et al., 2010; Espunya et al., 2012; Corpas et al., 2013; Frungillo et al., 2014). GSNO level is regulated either by its production or by an enzymatic turnover mechanism catalyzed by GSNO reductase (GSNOR). Mutations in *GSNOR* gene have been shown to cause pleiotropic plant growth defects, impaired plant disease responses, heat sensitivity, and resistance to cell death (Feechan et al., 2005; Rusterucci et al., 2007; Lee et al., 2008; Kwon et al., 2012; Xu et al., 2013). The *gsnor-ko* plants contain elevated amount of *S*-nitrosothiols (SNO) and nitroso species indicating that GSNOR activity controls the level of both GSNO and indirectly protein-SNOs (Liu et al., 2001; Feechan et al., 2005; Lee et al., 2008). GSNOR, originally identified in plants and other organisms as a glutathione-dependent formaldehyde dehydrogenase (GS-FDH), belongs to the class III alcohol dehydrogenase family (EC 1.1.1.1) (Martinez et al., 1996). The crystal structure of GS-FDH from mammals, yeast and plants revealed that the enzyme is a homodimer coordinating two zinc atoms per subunit (Sanghani et al., 2002; Kubienova et al., 2013). Few years later, evidence was provided that GS-FDH is involved also in the *S*-nitrosothiol metabolism (Liu et al., 2001) and GSNO degrading activity was described for *Arabidopsis* GS-FDH (Sakamoto et al., 2002). GSNOR is a highly conserved enzyme in mammals, yeast and plants and is essential to protect cells under nitrosative stress (Liu et al., 2001; Corpas et al., 2011).

Reactive oxygen species as oxidants and signaling molecules have a fundamental influence in almost all biological processes (Apel and Hirt, 2004). The regulated production of ROS due to biotic and abiotic stimuli is necessary to activate downstream responses (Shaikhali et al., 2012; Foyer and Noctor, 2013; Dietz, 2014). However, the excessive accumulation of ROS can lead to detrimental consequences; therefore, precise regulation of ROS level is highly important. Next to the enzymatic decomposition of ROS by catalase, ascorbate peroxidase (APX) or other enzymes in the glutathione-ascorbate cycle, the non-enzymatic way by low molecular weight antioxidants, like glutathione (GSH) and ascorbate has crucial role to balance cellular redox changes (Foyer and Noctor, 2013). ROS can modify cysteine thiols and methionine residues of redox sensitive target proteins resulting in oxidative posttranslational modifications or irreversible oxidations of proteins (Konig et al., 2012; Waszczak et al., 2015). It has been shown that these oxidative modifications affect enzyme or metal-binding activity of important signaling proteins, like protein phosphatases and mitogen-activated protein kinases (Gupta and Luan, 2003; Jammes et al., 2009; Waszczak et al., 2014) or transcription factors (Dietz, 2014).

H_2O_2 and NO are commonly produced during various stress conditions suggesting a strong interplay between both signaling molecules. NO accumulation induced by *Verticillium dahlia* toxin depends on prior H_2O_2 production (Yao et al., 2012). Further evidences supported cross-talks of ROS and NO in cryptogein-induced defense response of tobacco cells (Kulik et al., 2014) and also in systemic acquired resistance in *Arabidopsis* (Wang et al., 2014). H_2O_2-induced NO production mediates abscisic acid-induced activation of a mitogen-activated protein kinase cascade (Zhang et al., 2007) and contributes to hydrogen-promoted stomatal closure (Xie et al., 2014). Despite the evidences of the crosstalk of ROS and NO signaling, there are still gaps in that regard how they control each other level and what is the consequence of their interactions.

Therefore, the focus of this study was to investigate a direct impact of ROS on GSNOR protein and thereby on cellular NO metabolism. We show that GSNOR activity is inhibited by paraquat-induced oxidative burst in wild type *Arabidopsis* seedlings accompanied by an increased cellular *S*-nitrosothiol and nitrite level. Furthermore, *gsnor* plants accumulate GSH, which acts as redox buffer to scavenge RNS. Transcripts encoding for redox-related proteins and activities of GSH-dependent enzymes were increased. Furthermore, we measured GSNOR activity under oxidizing conditions and analyzed cysteine residues by LC-MS/MS for potential oxidative modifications. We demonstrated that oxidative conditions inhibited GSNOR activity *in vitro* and this inhibition correlated with Zn^{2+} release of GSNOR. In sum, ROS-dependent regulation of GSNOR contributes to fine-tuning of NO/SNO levels, which can act directly as a ROS scavenger and/or activate antioxidant mechanisms in response to oxidative stress.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana (L.) Heynh (ecotype Columbia-0 and Wassilewskija) wild type seeds and knock-out mutants of the *GSNOR* gene (At5g43940) were obtained from GABI-kat (GABI-Kat 315D11, background Columbia-0) and FLAG T-DNA collections (Versailles Genomic Resource Centre; FLAG_298F11, background Wassilewskija). After vernalisation for 2 days (4°C in dark), plants were cultivated for 4 weeks in a climate chamber at 60% relative humidity under long-day condition (16 h light/8 h dark cycle, 20°C day/18°C night regime, 100 μmol m^{-2} s^{-1}

photon flux density). For seed germination analyses, *Arabidopsis* seeds were surface sterilized and grown on half strength MS medium containing 1% sucrose in a climate chamber under long-day condition.

Paraquat Treatment

Sterile seeds were germinated and grown on half strength MS plates containing different paraquat (methyl viologen, Sigma–Aldrich, Steinheim, Germany) concentrations (0.25–10 μM) for 1 to 2 weeks. To test tyrosine nitration, Western blot was made using anti-nitrotyrosine antibody (Merck Millipore, Darmstadt, Germany) as described (Holzmeister et al., 2015). For spray application, 1, 10, and 50 μM paraquat or water (control) was sprayed onto the leaf surface of 4-week-old plants. Leaves were collected after 1-day of treatment, frozen and kept at −80°C until use. For Western-blot analysis of total protein extract made from paraquat-treated leaves, polyclonal antibody against *Arabidopsis* GSNOR (Agrisera, Sweden) was used.

NO Fumigation

Four-week-old plants were placed in an incubator and fumigated with 80 ppm gaseous NO or with synthetic air without NO for 20 min. The experimental setup consisted of controlled-environment cabinets as well as equipment to adjust and control gaseous NO treatment. NO concentration was monitored with a Chemiluminescence Nitrogen Oxides Analyzer AC32M (Ansyco, Karlsruhe, Germany). After fumigation, the plants were placed in the growth chamber until sample collection.

Cloning of AtGSNOR and Cysteine Mutants GSNOR^{C47S}, GSNOR^{C177S}, GSNOR^{C271S}

Total RNA isolated from wild type *Arabidopsis* leaves was used to produce cDNAs by SuperScript II Reverse transcriptase (Invitrogen, Carlsbad, CA, USA). For amplification of coding sequence of AtGSNOR for gateway cloning, the first PCR reaction was made using gene-specific primers (ADH2-ATG-for: 5′-ATGGCGACTCAAGGTCAG-3′; ADH2-TGA-rev: 5′-TCA TTTGCTGGTATCGAGGAC-3′). Afterward, the second PCR reaction was performed to introduce recombination sequences (att) at the 5′- and 3′-end using the following primers (ADH2-GW-forward: 5′-GGGGACAAGTTTGTACAAAAAAG CAGGCTTCATGGCGACTCAAGGTC-3′; ADH2-GW-reverse: 5′- GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATTTG CTGGTATCGAG-3′). The resulting PCR products were cloned into pDONR221 vector (Invitrogen, Carlsbad, CA, USA) by recombination reaction using BP Clonase enzyme mixture according to the instructions of the manufacturer. After sequencing, the correct clone was transferred into the expression vector pDEST17 by recombination using LP Clonase enzyme mixture (Invitrogen, Carlsbad, CA, USA).

Site-Directed Mutagenesis

The modification of single nucleotide residues was performed as previously described (Lindermayr et al., 2003). Briefly, for mutation, a complementary pair of oligonucleotides was synthesized harboring the desired alterations. For amplification, 20 ng plasmid DNA was used in a total volume of 15 μl, including 1 μM each primer, 200 μM dNTPs, and 1 U of PfuTurbo DNA polymerase. After denaturation (2 min at 94°C) 18 cycles were conducted, consisting of 45 s at 94°C, 30 s at 55°C, and 15 min at 72°C, followed by a final extension step at 72°C for 10 min. Subsequently, the parental and hemi-parental DNA was digested with DpnI and the amplified plasmids were transformed into *Escherichia coli* DH5α. The mutation was verified by sequencing. The next primers were used to make GSNOR^{C47S} forward: CTACACTGCTCTTAGTCACACCGACGCTTAC and reverse: GTAAGCGTCGGTGTGACTAAGAGCAGTGTAG; for GSNOR^{C177S} forward: GTTTGCCTTCTTGGAAGTGGTGTTC CCACTG and reverse: CAGTGGGAACACCACTTCCAAGA AGGCAAAC; for GSNOR^{C271S} forward: GACTACAGCTTTGA GAGCATCGGGAATGTCTC and reverse: GAGACATTCCCG ATGCTCTCAAAGCTGTAGTC.

Purification of Recombinant GSNOR and Cysteine Mutants

For expression of the recombinant N-terminal His_6 fusion proteins the wild type pDEST17-GSNOR and the cysteine mutants GSNOR^{C47S}, GSNOR^{C177S}, GSNOR^{C271S} were transformed into the *E. coli* strain BL21 DE3 pLysS., The LB cultures at A_{600} ∼0.6 were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside and further incubate for 4 h at 28°C. After induction the bacterial cells were harvested by centrifugation and stored frozen. For protein isolation the cells were resuspended in a lysis buffer [25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% Triton X-100, 20% (v/v) glycerol, 1 mM β-mercaptoethanol] and disrupted by sonication. Cellular debris was removed by centrifugation and the soluble fraction was purified by affinity chromatography using Ni-NTA agarose (Qiagen, Hilden, Germany). Adsorbed proteins were eluted from the matrix with elution buffer containing 25 mM Tris-HCl pH 8.0, 250 mM imidazole, 10 mM DTT, 20% (v/v) glycerol. The eluates were aliquoted, frozen in liquid nitrogen and stored at −80 °C until analysis. The purity was checked by SDS-PAGE and the protein concentration was measured by Bradford assay (Bio-Rad). Before the further use the elutions were desalted by Zeba-Spin column (Thermo Scientific, Rockford, IL, USA).

Static Light-Scattering Analysis

Static light scattering (SLS) experiments on recombinant GSNOR were performed at 30°C using a Viscotek TDA 305 triple array detector (Malvern Instruments) downstream to an Äkta Purifier (GE Healthcare) equipped with an analytical size exclusion column (Superdex 200 10/300 GL, GE Healthcare) at 4°C. GSNOR protein was purified under reducing condition, followed by Äkta purification, than 200 μg GSNOR was oxidized by 1 mM H_2O_2. The reduced and oxidized GSNOR samples were run in 50mM Tris-HCl pH 8.0, 200 mM NaCl, with or without 10 mM DTT, respectively, at a flow rate of 0.5 ml/min. The molecular masses of the samples were calculated from the refractive index and right-angle light-scattering signals using Omnisec (Malvern Instruments). The SLS detector was calibrated with a 4 mg/ml

BSA solution with 66.4 kDa for the BSA monomer and a dn/dc value of 0.185 ml/g for all protein samples.

Enzyme Activity Assays

Purified GSNOR protein was re-buffered using Zeba Spin column equilibrated with 20 mM Tris-HCl pH 8.0 buffer. GSNOR activity was determined by measuring the reaction rate of NADH usage at 340 nm in Ultrospec 3100 pro (Amersham Biosciences) spectrophotometer. The reaction buffer contained 20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.2 mM NADH. Serial dilutions (50–2000-fold) of recombinant His-tagged GSNOR proteins (wild type and cysteine mutants) were prepared and the reaction was started to add GSNO (Enzo Life Sciences) at a final concentration of up to 0.5 mM. Water was used instead of GSNO in the reference sample. The reaction was monitored for 5 min and the linear rate was corrected with a reference rate without GSNO. There was no detectable NADH oxidation without enzyme. Specific activity was calculated using a molar extinction coefficient for NADH 6.22 $mM^{-1}cm^{-1}$. Effect of H_2O_2, PN or NEM on GSNOR activity was analyzed by incubation of recombinant GSNOR with these compounds for 20 min in 20 mM Tris-HCl pH 8.0. Excess H_2O_2, PN or NEM was removed by gel filtration using Zeba desalting columns (Thermo Fisher Scientific, Waltham, MA, USA). Zeba Spin columns were equilibrated with 20 mM Tris-HCl pH 8.0. The eluates were used to determine GSNOR activity. To analyze reversibility of H_2O_2-dependent inhibition of GSNOR, excess H_2O_2 was removed (Zeba Spin, 20 mM Tris-HCl pH 8.0) and inhibited GSNOR was divided into two fractions. One fraction was treated with water, the other one was treated with 10 mM DTT for 10 min at room temperature. Before measuring the activity, both samples were desalted using Zeba Spin columns equilibrated with 20 mM Tris-HCl pH 8.0. To analyze the effect of H_2O_2 in presence of excess Zn^{2+}, GSNOR was treated with 0.5 mM H_2O_2 in presence of 0.5 μM $ZnSO_4$ for 20 min. Excess H_2O_2 and $ZnSO_4$ was remove using Zeba Spin columns (20 mM Tris-HCl pH 8.0) and GSNOR activity was determined.

To measure GSNOR activity from plant tissue, total soluble proteins were extracted from treated seedlings or leaves in buffer of 0.1 M Tris–HCl pH 7.5, 0.1 mM EDTA, 0.2% TritonX-100, 10% glycerol. The homogenate was centrifuged twice at 14.000 *g* for 20 min at 4°C and total protein concentration of the supernatant was measured according to Bradford using BSA as a standard. GSNOR activity was determined by incubating 100 μg of protein extract in 1 ml reaction buffer as described above.

Glutathione reductase activity assay is based on the NADPH-dependent reduction of GSSG to GSH. GR activity was measured by the rate of NADPH oxidation at 340 nm. Proteins from 2-week-old seedlings were extracted with extraction buffer (50 mM potassium phosphate pH 7.8, 0.1 mM EDTA, 0.5% Triton X-100, 0.5% PVP-40). 50 μg total protein was incubated in 1 ml GR reaction buffer (100 mM potassium phosphate pH 7.8, 1 mM EDTA, 0.2 mM NADPH). The reaction was started by addition of 100 μl of 5 mM GSSG and was monitored at 340 nm for 5 min. The linear rate of reaction was corrected with a reference rate without GSSG (molar extinction coefficient for NADPH 6.22 $mM^{-1}cm^{-1}$).

Glutathione-*S*-Transferase (GST) activity was measured by spectrophotometrically using the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB) for GSH attachment. 50 μg of total protein was incubated in 1 ml GST reaction buffer (100 mM potassium phosphate pH 7.8, 1 mM CDNB in 80% ethanol) and the reaction was started by adding 100 μl of 10 mM GSH. The production of GSH-CDNB conjugate was monitored at 340 nm [molar extinction coefficient (ε) = 9600 M^{-1} cm^{-1}].

Zn^{2+} Release Assay

Free Zn^{2+} ions were detected by a metallochromic indicator 4-(2-pyridylazo)-resorcinol (PAR), which binds Zn^{2+} in a 2:1 complex and turn its color from yellow to orange with strong absorbance at 490 nm (Crow et al., 1997). Recombinant GSNOR (50–100 μg) was oxidized by increasing molar excess (100–3000 to protein) of H_2O_2 for 1 h in 50 mM Tris-HCl pH7.2 and mix with 100 μM PAR. The absorbance of PAR_2-Zn complex was measured at 490 nm and the Zn content was calculated using $ZnCl_2$ standard curve.

Mass Spectrometric Analyses of GSNOR

Twenty microliter of H_2O_2-treated (molar ratio of H_2O_2 to protein was 100:1 or 1000:1) or water-treated (as control) recombinant GSNOR (corresponding to approximately 8 μg of protein) was incubated with 20 μl of non-reducing SDS-gel loading buffer (20% glycerol, 4% SDS, 0.125 M Tris-HCl pH 6.8) containing 55 mM 2-iodoacetamide (IAA). To remove the excess of IAA completely, samples were separated under non-reducing conditions on SERVAGel TG PRiME 4-12% (SERVA, Heidelberg, Germany). Then the gel was stained with Coomassie overnight and after de-staining the bands were excised and transferred into 1.5 mL reaction tubes. Reduction of cysteine residues was performed for 30 min at 55°C using 45 mM DTT in 50 mM NH_4HCO_3. After removal of DTT solution, blocking was performed using 50 mM *S*-methyl-methanethiosulfonate (MMTS) for 30 min, then the gel slices were washed three times using 50 mM NH_4HCO_3. The in-gel digestion was performed overnight using 500 ng bovine chymotrypsin (Roche Diagnostics, Mannheim, Germany) or 70 ng trypsin (Promega, Fitchburg, WI, USA). Peptides were separated on NanoLC Ultra chromatography system (Eksigent, Redwood City, CA, USA) coupled to an LTQ Orbitrap XL mass analyzer (Thermo Fisher Scientific, San Jose, CA, USA). Mobile phase A was 0.1% formic acid and mobile phase B was 84% acetonitrile/0.1% formic acid. For separation, a reversed phase nano-column (Reprosil-Pur C18 AQ, 2.4 μm; 150 mm × 75 μm, Dr. Maisch, Ammerbuch-Entringen, Germany) at a flow rate of 280 nl/min was used. The separation method consisted of two linear gradients (1–30% B in 120 min and 30–60% B in 10 min). Mass spectra were acquired in cycles of one MS Orbitrap scan, followed by five data dependent ion trap MS/MS scans (CID, collision energy of 35%). MS spectra were searched using MASCOT 2.4 (Matrix Science, London, UK) using the *A. thaliana* subset of the SwissProt Database and the following parameters: (a) Variable modifications: Dioxidation (C), Trioxidation (C), Methylthio (C), Carbamidomethyl (C), Oxidation (M); (b) Enzyme: none, (c) Peptide charge: 1+, 2+, and 3+; (d) Peptide tol. ± : 10 ppm; (e) MS/MS tol. ± :

0.8 Da. MASCOT DAT files were imported into the Scaffold software package (Proteome Software Inc., Portland, OR, USA) and filtered for hits with a confidence of 99% at the protein level and 95% for individual peptides.

Microarray Analysis

Total RNA from 4 to 5-week old rosette leaves of Wassilewskija *Arabidopsis* WT and *gsnor* was isolated using RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Quality checking and quantification of RNA isolates were carried out using Agilent RNA 6000 Nano kit on Agilent 2100 BioAnalyzer. Microarray analysis was performed on Agilent platform using the technique "One color Microarray-based Gene Expression Analysis" according to the protocol described in the Agilent manual. The raw expression data of three biological replicates per genotype was analyzed using GeneSpring GX software tool. Statistical analysis were carried out to identify the differentially expressed genes ($p < 0.05$) between the two genotype using One Way Anova analysis with the Benjamini-Hochberg multiple test correction (FDR) and SNP *Post hoc* test. From the gene list, those ones regulated at least by twofold differences were selected for downstream analysis. Microarray data are available in the ArrayExpress database[1] under accession number E-MTAB-4756.

Determination of Glutathione

The amount of the reduced and oxidized glutathione was determined in 2-week-old seedlings using the GR-based recycling assay described previously (Queval and Noctor, 2007). Furthermore, total glutathione, cysteine and γ-glutamyl-cysteine content were measured by reverse-phase HPLC after NO fumigation experiment. Briefly, 200 mg leaf material was extracted in 0.1 M HCl. Subsequently, all low molecular weight thiols were reduced by addition of DTT and then derivatized with 10 mM monobromobimane as described previously (Wirtz et al., 2004). Samples were analyzed by reverse-phase HPLC and fluorescence excitation at 380 nm.

Determination of Ascorbate

The amount of the reduced and total ascorbate was determined as described by Queval and Noctor (2007). Four weeks old *Arabidopsis* plants were sprayed with 0, 1, 10, or 50 μM of paraquat, harvested and stored in −80°C until use. The amount of reduced ascorbate (ASC) was measured at 265 nm in a Tecan plate reader before and after incubation with ASC oxidase. ASC oxidase converts the reduced ASC to the non-absorbing oxidized form. For determination of total ASC, the oxidized ASC was first reduced to ASC by adding 1 mM DTT for 30 min, then total ASC was measured as above. ASC standard was used to calculate the amount of ASC.

In situ Staining of Diaminobenzidine (DAB) and Nitroblue Tetrazolium (NBT)

For detection of H_2O_2, paraquat or water treated *Arabidopsis* seedlings were vacuum infiltrated with 0.1% 3,3′-Diaminobenzidine (DAB) in 10 mM MES pH 6.5 solution, washed three times with water and incubated for 45 min at RT in light. After staining, plants were destained with 90% ethanol at 60°C. The brown precipitate shows the presence of H_2O_2 in the cell and tissue.

Arabidopsis plants were vacuum infiltrated with nitroblue tetrazolium (NBT) [50 mM potassium phosphate/pH 6,4; 10 mM NaN_3; 0,1% (w/v) NBT] solution, incubated for 45 min in dark and washed three times with water. Afterward, plants were de-stained with 90% ethanol at 60°C.

Determination of Nitrosothiols and Nitrite

Total nitrite, nitrate, and nitrosothiol content were measured using a Sievers 280i nitric oxide analyser (GE Analytical Instruments, Boulder, CO, USA). Proteins were extracted from rosettes using extraction buffer (137 mM NaCl, 0,027 mM KCl, 0,081 mM $Na_2HPO_4.2H_2O$, 0,018 mM NaH_2PO_4). Leaf protein extract was injected into the purging vessel containing 3.5 ml of acidified KI/I_3^- solution (reducing agent) at 30°C. The recorded mV signals were plotted against a calibration curve produced using known concentrations of sodium nitrite solution to quantify the nitrite level. To estimate the *S*-nitrosothiol content (RSNO), the above protocol was repeated by pre-treating the leaf protein extract with 20 mM sulphanilamide (in 1 M HCl) at the ratio of 9:1. For nitrate quantification, the reducing agent was replaced with vanadium chloride at 95°C. The recorded mV signals were plotted against a calibration curve produced using known concentrations of sodium nitrate solution to quantify the nitrate levels.

Statistical Analysis

For multiple comparisons, analysis of variance was performed by Anova (one way or two way) followed by Holm-Sidak test. For pairwise comparison, Student's *t*-test was used. The level of significance is indicated in each figure.

RESULTS

Paraquat-Induced Inhibition of GSNOR Activity

S-nitrosoglutathione-reductase controls intracellular levels of SNOs and thereby this enzyme is important for NO homeostasis. Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) is an herbicide, which induces the accumulation of ROS, such as superoxide, H_2O_2 and other deleterious oxygen radicals (Dodge, 1971; Babbs et al., 1989) and is a good tool to investigate the effect of ROS on GSNOR activity *in vivo*. Four week old *Arabidopsis* plants (Col-0) were sprayed with this herbicide. Paraquat treatment for 24 h significantly decreased GSNOR activity in a dose-dependent manner (**Figure 1A**). Application of 50 μM paraquat resulted in 40% enzyme inhibition, which could be restored with 10 mM of the reducing agent 1,4-dithiothreitol (DTT) suggesting that oxidative modification(s) are responsible for inhibition of GSNOR activity. Moreover, immunoblot analysis using GSNOR-specific antibody demonstrated only

[1] http://www.ebi.ac.uk/arrayexpress

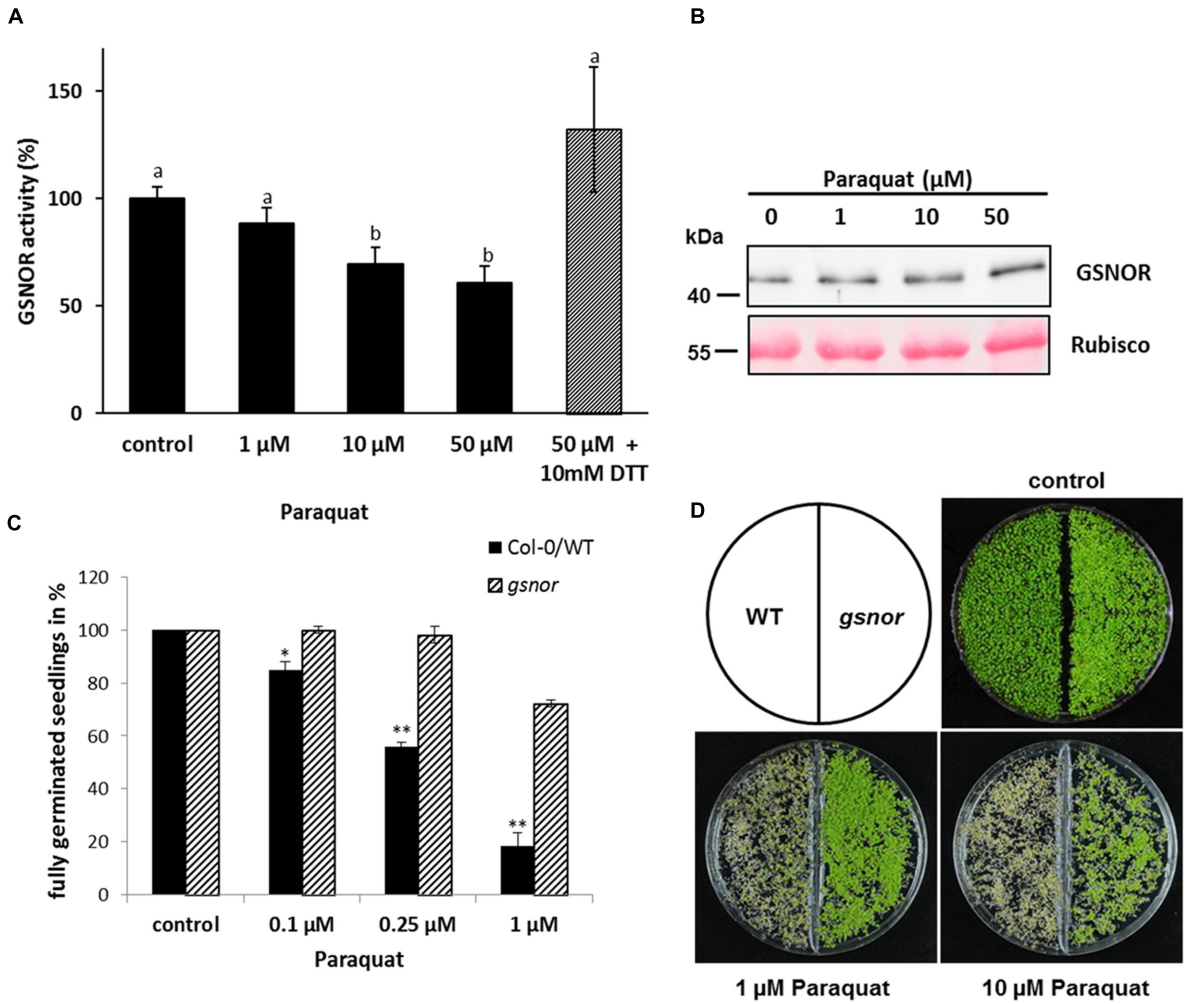

FIGURE 1 | Inhibition of GSNO-reductase (GSNOR) activity *in vivo* by paraquat and tolerance of *gsnor* mutants to oxidative stress. (A) Measurement of GSNOR activity from crude leaf extracts of 4-week-old *Arabidopsis* plants exposed to different paraquat concentrations for 24 h. For restoring enzyme activity, 10 mM DTT was added to the extract before the measurement (gray bar). Values are expressed as percentage of water treated control plants (specific activity varies 49.2–91.1 nmol NADH min^{-1} mg^{-1}) and represent the mean ± SD calculated from three biological replicates. Different letters indicate significant differences, $p < 0.05$, Anova. **(B)** Western blot of paraquat-treated plant extracts using GSNOR-specific antibody. Ponceau staining of Rubisco protein represents the equal loading. **(C)** Germination rates of 1-week-old Col-0 WT and *gsnor* mutant growing on 0.1, 0.25, and 1 µM paraquat-containing media. The germination rate was calculated by counting fully germinated seedlings with open cotyledons and is presented in percentage of control (without paraquat). $*p < 0.05$, $**p < 0.005$ indicate significant differences between WT and *gsnor*. **(D)** Representative pictures of WT and *gsnor* seedlings germinated on paraquat-containing media. Control is without paraquat.

a slight change of GSNOR protein amount during paraquat treatment (**Figure 1B**). The accumulation after treatment with 50 µM paraquat is around 1.3-fold in comparison to the control sample and might partly compensate for the paraquat-induced inhibition of GSNOR.

To study the physiological function of paraquat/ROS-induced GSNOR inhibition, plants lacking GSNOR function were analyzed for their response to paraquat treatment. Interestingly, Chen et al. (2009) previously observed that GSNOR plays a role in regulating paraquat-induced cell death in plant cells through modulating intracellular NO level. We used two T-DNA insertion alleles for *GSNOR* (background Col-0 and Wassilewskija, named *gsnor* and WS/*gsnor*, respectively) to test their germination and growth in presence of paraquat. Seeds of WT and *gsnor* plants were cultivated on MS media containing 0–1 µM paraquat and the germination rate was determined by counting fully germinated seedlings with two open cotyledons. The germination rate of WT plants was strongly reduced to 20% in presence of 1 µM paraquat (**Figure 1C**). In contrast, the germination rate of *gsnor* plants was significantly higher (72%) at this paraquat concentration (**Figure 1C**). Similar results could be observed using the T-DNA insertion line in Wassilewskija background (WS/*gsnor*; Supplementary Figure S1A). Paraquat induced cell death phenotype of WT seedlings was obvious using

higher paraquat concentration (1 and 10 μM) by the yellowish-brown colored cotyledons and restricted growth (**Figure 1D**). Interestingly, *gsnor* mutant showed an enhanced tolerance even in the presence of 10 μM paraquat demonstrated by green viable seedlings.

Paraquat-induced O_2^- can react with NO resulting in $ONOO^-$ production, which can oxidize cysteine residues or nitrate tyrosine residues of proteins. Protein tyrosine nitration is a marker for pathological processes in cell death. WT seedlings germinated on 0.5 μM paraquat showed stronger tyrosine nitration than *gsnor* seedlings (Supplementary Figure S1B) suggesting a weaker cell death phenotype for *gsnor* mutant, which correlates with the observed visible effect of more green seedlings (**Figure 1D**).

Paraquat-Induced Changes in NO Metabolism

Since GSNOR activity is important for SNO homeostasis, intracellular SNO levels were analyzed after paraquat treatment. WT and *gsnor* plants were sprayed with 1, 10, and 50 μM paraquat and total cellular SNO content was determined. SNO levels increased in paraquat-treated WT plants from 15 pmol mg^{-1} up to 40 pmol mg^{-1} (50 μM paraquat) (**Figure 2A**). The *gsnor* mutant has already elevated SNO content under control condition (around 40 pmol mg^{-1}) correlating to the loss of GSNOR function. Paraquat application did not result in significant further accumulation of SNOs in *gsnor* plants (**Figure 2A**). In the living cell NO can be oxidized to give nitrite, which would indicate freshly produced NO. Therefore, we measured nitrite content from WT and *gsnor* plants treated with paraquat. The nitrite level increased in both plant lines in a similar degree in the presence of increasing herbicide concentrations (**Figure 2B**); however, the nitrite content was significantly higher over the treatment in *gsnor* plants.

gsnor Plants Have Higher GSH-Dependent Antioxidant Capacity

Resistance against oxidative stress is often related to an enhanced cellular antioxidant capacity. Therefore, the amount of reduced and oxidized glutathione (GSH and GSSG, respectively) was measured in WT and *gsnor* seedlings germinated on media with and without 0.5 μM paraquat for 14 days. GSH level of untreated *gsnor* plants was about twofold higher than of WT plants and the content increased upon paraquat treatment about four and threefold in WT and *gsnor* plants, respectively (**Figure 3A**). About 10% of the glutathione pool was oxidized under control conditions in both plant lines demonstrating that the redox status is the same in WT and *gsnor* plants (GSH:GSSG ratio, **Figure 3A**). Germination on paraquat-containing media for 14 days resulted in increased total glutathione content in both plant lines; however, the glutathione pool was more reducing in the *gsnor* mutant than in WT (GSH:GSSG ratio around 30 and 20, respectively, **Figure 3A**). This result indicates that *gsnor* may cope better with a long-term paraquat treatment than WT plants to keep cellular redox condition more reducing.

In correlation with the increased level of glutathione, we measured around 20% higher activity for glutathione reductase (GR) enzyme in untreated *gsnor* plants in comparison to WT plants (**Figure 3B**). GR reduces GSSG to GSH in a NADPH-dependent manner. In both lines, GR activity increased by around 30% after paraquat treatment. The glutathione *S*-transferase (GST) activity was also higher (with 15%) in untreated *gsnor* plants than in WT and the GST activity increased in both plant lines after paraquat-treatment by around 10% (**Figure 3B**). Ascorbate is another important cellular reductant inter-connected to the anti-oxidative response in chloroplast. However, although the levels of reduced ascorbate were higher in *gsnor* plants after paraquat-treatment, the differences were significant only at high paraquat concentrations (Supplementary Figure S2). All these results suggest that the enhanced GSH levels and enhanced activities of GSH-dependent enzymes contribute to the paraquat tolerant phenotype of *gsnor* plants.

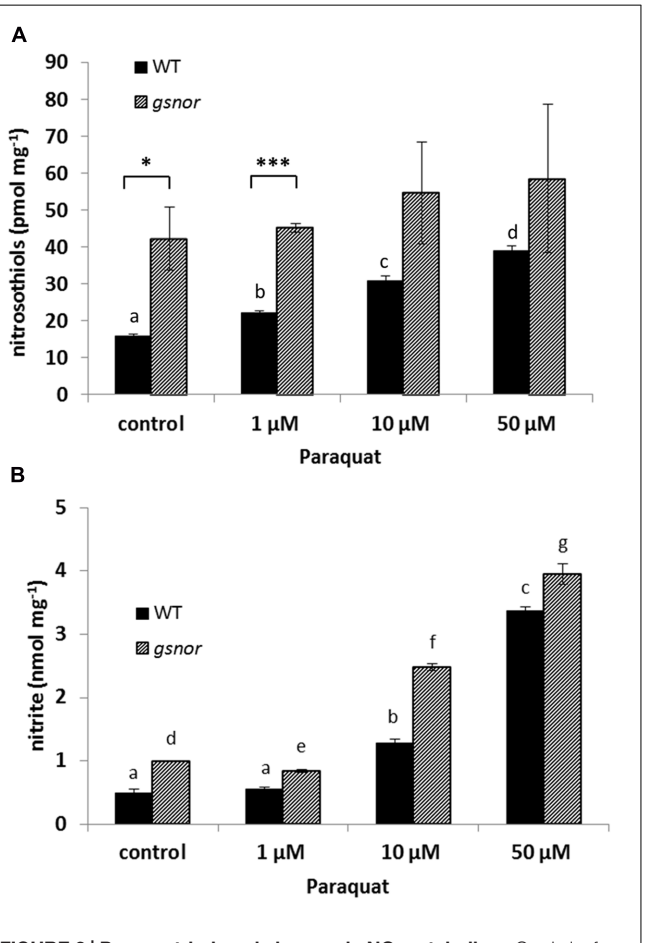

FIGURE 2 | Paraquat-induced changes in NO metabolism. Crude leaf extracts from 4-week-old WT (black bar) and *gsnor* (gray bar) plants exposed to different paraquat concentrations (1, 10, and 50 μM) for 24 h were used for determination of nitrosothiol **(A)** and nitrite **(B)** contents. Values were normalized against total protein content and represent the mean ± SD calculated from three replicates. The different letters indicate significant differences among the samples ($p < 0.05$, Anova) and *$p < 0.05$, ***$p < 0.001$ represent significant differences between WT and *gsnor* (a).

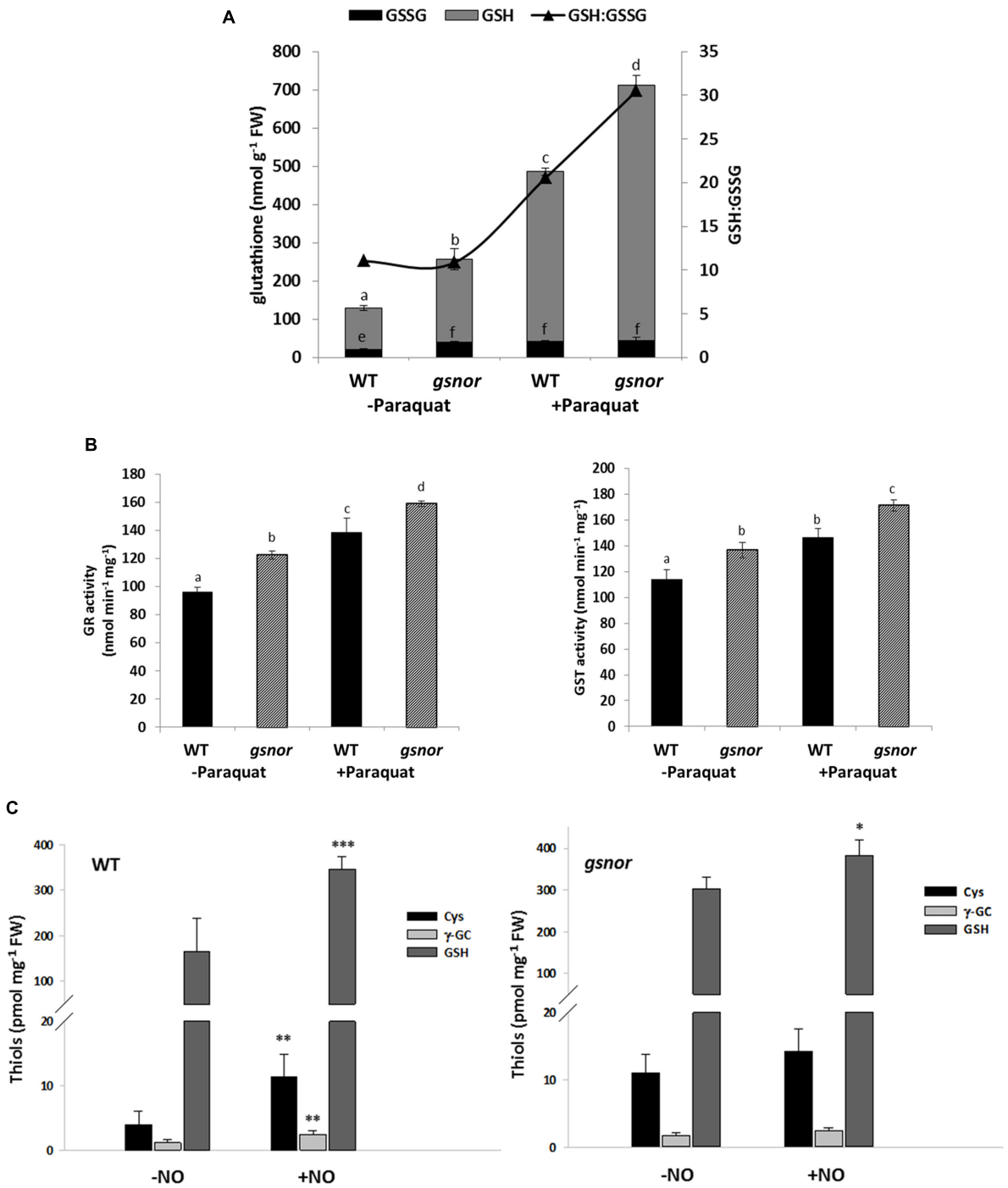

FIGURE 3 | *gsnor* plants has an elevated glutathione-dependent antioxidant capacity to cope with oxidative stress. (A) Determination of reduced (GSH) and oxidized (GSSG) glutathione from crude extracts of 14-day-old WT and *gsnor* plants were grown on MS media with and without 0,5 μM paraquat. The values were normalized against fresh weight (FW). The ratio of GSH to GSSG (GSH:GSSG, black triangle) is presented on the right axis. **(B)** Enzyme activities for glutathione reductase (GR, left panel) and glutathione-*S*-transferase (GST, right panel) were determined from the same treatments as in **(A)**. The values of enzyme activity represent the mean ± SD calculated from three biological replicates. The different letters indicate significant differences among the samples ($p < 0.05$, Anova). **(C)** Analysis of thiol-containing intermediates of the GSH biosynthesis pathway by HPLC. Four week-old WT plants (left panel) and *gsnor* mutants (right panel) were fumigated with and without 80 ppm NO gas for 20 min. After 1 h of regeneration rosettes were harvested for determination of cysteine (Cys), γ-glutamylcysteine (γ-GC), and total glutathione (GSH) content. Values were normalized against total fresh weight and represent the mean ± SD calculated from three to five samples of each line. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ represent significant differences between control (−NO) and NO-treated samples (*t*-test).

TABLE 1 | Candidate genes involved in H_2O_2 metabolism.

Gene	Fold-change	Protein
Upregulated		
AT4G16270	13.66	Peroxidase 40 (PER40) (P40)
AT1G44970	12.88	Peroxidase, putative
AT5G47000	11.94	Peroxidase, putative
AT5G64100	5.31	Peroxidase, putative
AT1G14540	5.27	Anionic peroxidase, putative
AT5G64110	5.15	Peroxidase, putative
AT5G64120	4.13	Peroxidase, putative
AT5G39580	3.84	Peroxidase, putative
AT3G03670	3.80	Peroxidase, putative
AT5G05340	3.60	Peroxidase, putative
AT4G33420	2.95	Peroxidase, putative
AT5G06720	2.92	Peroxidase, putative
AT4G08770	2.23	Peroxidase, putative
AT4G37520	2.12	Peroxidase 50 (PER50) (P50) (PRXR2)
AT5G19890	2.09	Peroxidase, putative
AT5G15180	2.05	Peroxidase, putative
AT1G60740	18.77	Peroxiredoxin type 2, putative
AT1G65970	6.99	TPX2 (thioredoxin-dependent peroxidase 2)
AT1G69880	6.20	ATH8 (thioredoxin H-type 8)
AT5G61440	3.31	ACHT5 (ATYPICAL CYS HIS RICH THIOREDOXIN 5)
AT1G59730	2.48	ATH7 (thioredoxin H-type 7)
AT2G29490	33.42	ATGSTU1 (GLUTATHIONE S-TRANSFERASE TAU 1)
AT1G17180	32.23	ATGSTU25 (GLUTATHIONE S-TRANSFERASE TAU 25)
AT2G29480	28.71	ATGSTU2 (GLUTATHIONE S-TRANSFERASE TAU 2)
AT1G17170	11.78	ATGSTU24 (GLUTATHIONE S-TRANSFERASE TAU 24)
AT2G29470	5.73	ATGSTU3 (GLUTATHIONE S-TRANSFERASE TAU 3)
AT1G69930	5.61	ATGSTU11 (GLUTATHIONE S-TRANSFERASE TAU 11)
AT1G59670	4.15	ATGSTU15 (GLUTATHIONE S-TRANSFERASE TAU 15)
AT3G09270	3.94	ATGSTU8 (GLUTATHIONE S-TRANSFERASE TAU 8)
AT1G74590	3.74	ATGSTU10 (GLUTATHIONE S-TRANSFERASE TAU 10)
AT2G29440	3.65	ATGSTU6 (GLUTATHIONE S-TRANSFERASE TAU 6)
AT2G29420	3.41	ATGSTU7 (GLUTATHIONE S-TRANSFERASE TAU 7)
AT1G69920	2.38	ATGSTU12 (GLUTATHIONE S-TRANSFERASE TAU 12)
AT2G29460	2.31	ATGSTU4 (GLUTATHIONE S-TRANSFERASE TAU 4)
Downregulated		
AT4G11290	−2.04	Peroxidase, putative
AT4G31870	−2.24	ATGPX7 (glutathione peroxidase 7)
AT4G23340	−5.61	Oxidoreductase, 2OG-Fe(II) oxygenase family protein
AT1G55290	−3.28	Oxidoreductase, 2OG-Fe(II) oxygenase family protein
AT1G49390	−2.59	Oxidoreductase, 2OG-Fe(II) oxygenase family protein
AT2G44800	−2.36	Oxidoreductase, 2OG-Fe(II) oxygenase family protein

Gene expression analysis of redox-regulated genes by transcript profiling of gsnor under normal growing condition. Fold change ($p < 0.05$) represents transcript abundance in gsnor relative to WT plant.

Taken into consideration that loss of GSNOR function results in elevated SNO/NO and GSH levels, which are both important for resistance against paraquat-induced oxidative stress, we analyzed the interplay between both components. Four-week-old WT and *gsnor* plants were fumigated with 80 ppm NO gas for 20 min to mimic NO burst and levels of low molecular weight thiols of the GSH biosynthesis pathway such as cysteine, γ-glutamylcysteine and total glutathione were determined. NO fumigation resulted in an increase of these compounds in both plant lines (**Figure 3C**). In addition, untreated *gsnor* plants had around twofold higher levels of cysteine and glutathione than WT plants, indicating a connection between SNO/NO and the GSH biosynthesis pathway.

Genes Involved in Antioxidant Mechanisms Are Upregulated in *gsnor* Mutant

To further demonstrate the presence of a pre-induced antioxidant system in *gsnor* plants, a microarray analysis was performed of 4-week-old rosettes of *gsnor* and WT plants. Out of 2159 genes, which were differentially regulated, 1407 genes were significantly upregulated and 752 genes were significantly downregulated by at least twofold in *gsnor* mutant (Supplementary Table S1) (ArrayExpress accession number E-MTAB-4756). Gene enrichment analysis of the regulated genes using VirtualPlant 1.3 platform (Katari et al., 2010) revealed that the most significantly enriched functional categories of the upregulated genes were the catalytic-, hydrolase-, oxidoreductase-, and glutathione transferase-activities (Supplementary Table S2). Among these functional categories, we focused on genes related to processes metabolizing H_2O_2. Within the upregulated genes, 16 genes encoding for peroxidases were identified. Peroxidases are heme-containing enzymes that use hydrogen peroxide as the electron acceptor to catalyze a number of oxidative reactions (**Table 1**). Moreover, genes encoding for thioredoxins (H-type 7, H-type 8, and atypical ACHT5) and thioredoxin-dependent peroxidases were upregulated in *gsnor* plants (**Table 1**). The third subgroup of upregulated genes are encoding for 13 members of the Tau subfamily of GSTs (**Table 1**). The list of downregulated genes contains only a few transcripts related to redox-regulation, for example one putative peroxidase and a glutathione peroxidase 7 (**Table 1**). Moreover, four member of the Fe(II) and 2-oxoglutarate-dependent dioxygenase family with an oxidoreductase activity was found to be downregulated. In sum, the transcript profile analysis of *gsnor* plants suggests a pre-induced antioxidant system under normal growth condition, which can help to defend plants against subsequent oxidative stress.

In line with the transcriptional data, we have analyzed the O_2^- and H_2O_2 level in WT and *gsnor* plants after paraquat treatment. Ten days old seedlings grown on MS media were treated with 25 μM paraquat and vacuum infiltrated with either nitroblue tetrazolium (NBT) for O_2^- detection (Doke, 1983) or DAB for H_2O_2 accumulation (Thordal-Christensen et al., 1997) (Supplementary Figure S3). No obvious difference in O_2^- accumulation could be observed in paraquat-treated WT and *gsnor* plants (Supplementary Figure S3A). In contrast, H_2O_2 accumulation was lower in leaves of *gsnor* plants in comparison to WT plants (Supplementary Figure S3B) suggesting a higher capacity to metabolize H_2O_2 in *gsnor* line.

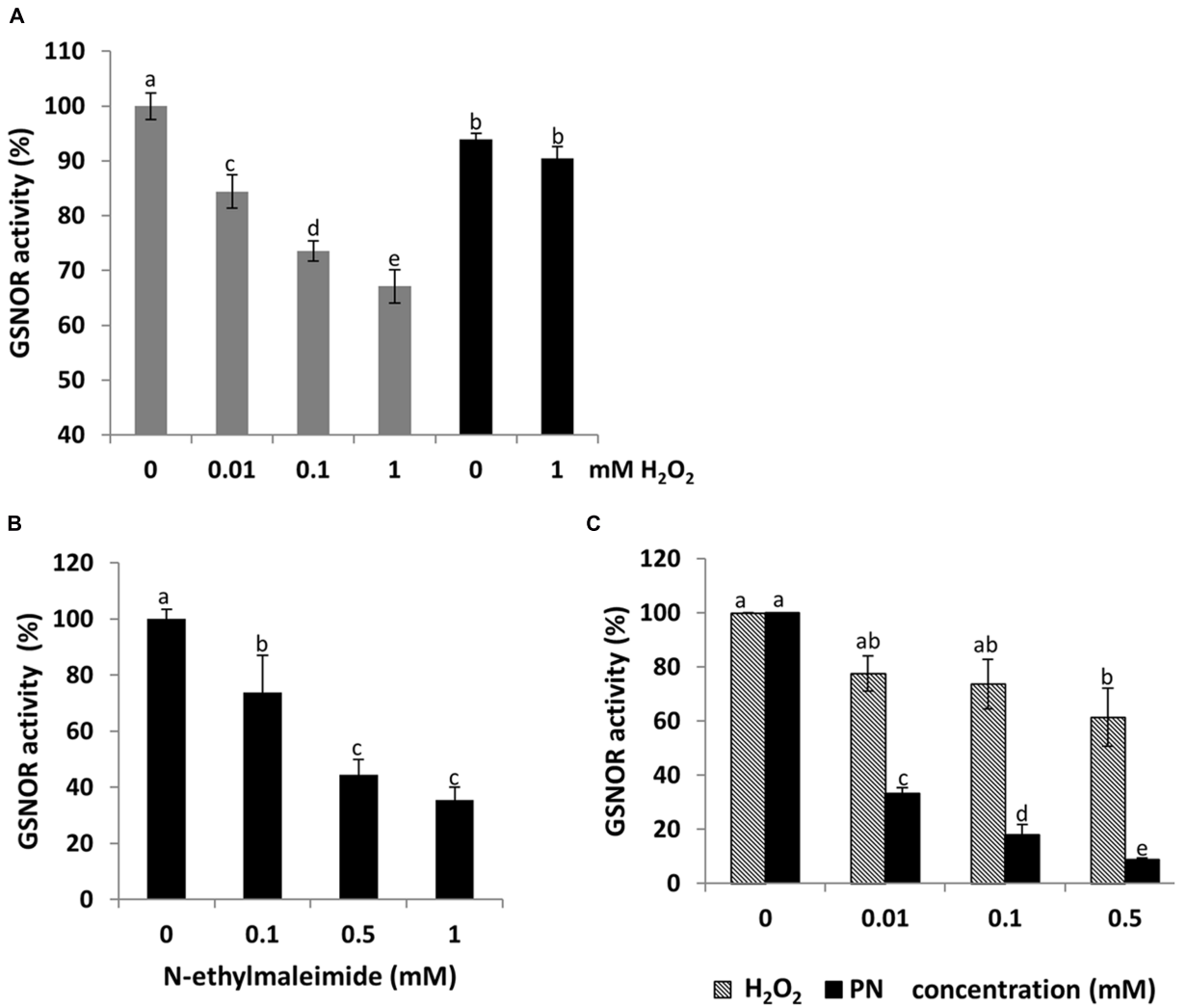

FIGURE 4 | Dose-dependent inhibition of GSNOR activity by oxidation *in vitro*. Measurements of GSNO reducing activity of GSNOR protein. Recombinant GSNOR (1 μg) was incubated with indicated concentrations of H_2O_2 **(A)**, N-ethylmaleimide **(B)**, and peroxynitrite (PN) and H_2O_2 **(C)**. For restoring GSNOR activity, 10 mM DTT was added to the H_2O_2 inhibited enzyme **(A)**. Values are expressed as percentage of the control activity (at 0 mM: 87.5–110.9 μmol NADH min^{-1} mg^{-1} varied among independent purifications) and represent the mean ± SD calculated from three independent assays. Different letters indicate significant differences ($p < 0.05$, Anova).

H_2O_2-Dependent Inhibition of GSNOR Activity *In vitro*

To analyze whether H_2O_2 affect *Arabidopsis* GSNOR activity, recombinant protein was incubated with increasing concentrations of H_2O_2 (0.01, 0.1, and 1 mM). A dose-dependent reduction of the GSNOR activity was observed (**Figure 4A**). Treatment with 10 μM H_2O_2 caused already 15% inhibition, which further decreased to 35% in the presence of 1 mM H_2O_2. Incubation of inhibited GSNOR with 10 mM of the reducing agent DTT partly restored the activity, concluding that enzyme inhibition was a result of reversible and irreversible modifications of one or several redox-sensitive amino acid residues. Treatment with the sulfhydryl-blocking agent *N*-ethylmaleimide also inhibited the activity of GSNOR to 40% demonstrating that cysteine residues are important for its activity (**Figure 4B**). Peroxynitrite ($ONOO^-$) is formed from the reaction of superoxide (O_2^-) and NO and acts as a potent oxidant on cysteine residues and as a nitrating agent on tyrosine residues of proteins (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011). Treatment of GSNOR with 10 μM of peroxynitrite reduced the activity by 70% (**Figure 4C**). Western blot analysis using 3-nitrotyrosine specific antibodies to detect tyrosine nitration of peroxynitrite-treated GSNOR protein did not show any nitration (data not shown) indicates that the peroxynitrite-dependent loss of GSNOR activity is probably due to the oxidation of cysteine(s) (Zeida et al., 2013).

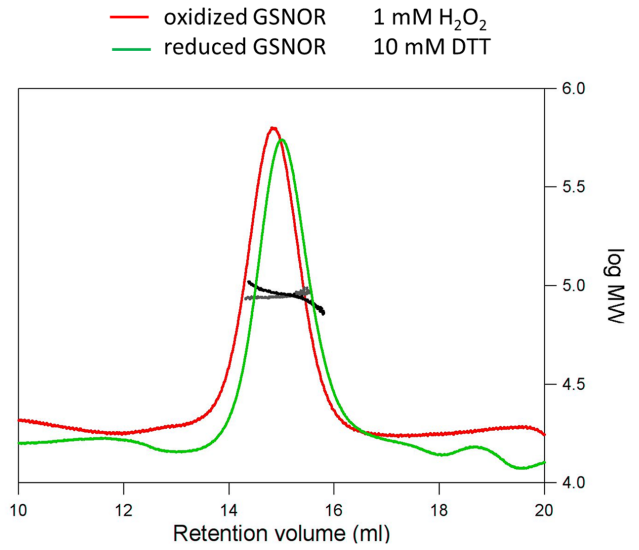

FIGURE 5 | Structural analysis of H_2O_2-treated GSNOR. Determination of the molecular weight of reduced (green) and oxidized (red) GSNOR using size exclusion chromatography in combination with static light scattering. The refractive index and right angle light scattering signals were monitored and used to determine the molecular weight of the reduced (black) and oxidized (gray) protein. 100 µl of the protein samples were applied to a Superdex 200 10/300 GL column. Reduced (10 mM DTT) and oxidized (1 mM H_2O_2) GSNOR were analyzed at approximately 1.5 mg/ml and both eluted as a homodimer of 90.1 and 88.4 kDa, respectively (calculated Mr is 86 kDa).

Structural Analysis of H_2O_2-treated GSNOR

As known from the crystal structure, GSNOR is a homodimer protein. To analyze, whether oxidative conditions cause changes in the native structure of GSNOR SLS experiments with reduced and oxidized recombinant GSNOR were performed. **Figure 5** shows that GSNOR protein is detected as dimer under both reducing (10 mM DTT) and oxidizing (1 mM H_2O_2) conditions with no significant difference between both states. The determined molecular weight was 90.1 and 88.4 kDa for reduced and oxidized GSNOR, respectively, which corresponds to the calculated weight of dimer (86 kDa for His_6-GSNOR).

Shifts in the electrophoretic mobility of proteins are diagnostic for the presence of oxidative modifications, like cysteine oxidations or formation of disulfide bridges (Benezra, 1994; Mahoney et al., 1996; Despres et al., 2003). Therefore, we treated recombinant GSNOR with 10–500 µM H_2O_2 and DTT and investigated its running behavior by non-reducing SDS PAGE (Supplementary Figure S4). We could not observe a defined shift in the mobility of the oxidized proteins, but the more diffuse protein bands with increasing concentration of H_2O_2 may indicate the presence of several different oxidative modifications. Subsequent treatment of oxidized GSNOR with 10 mM DTT resulted in a sharp single band demonstrating the reversibility of the oxidative modifications.

H_2O_2 Treatment of GSNOR Results in Oxidative Modification of Multiple Cysteine Residues

The thiol group of cysteine residues is susceptible to oxidation resulting in a formation of sulfenic, sulfinic, or sulfonic acids, the latter two are irreversible modifications. Moreover, disulfide bridges can be formed. Since GSNOR has 15 cysteine residues, we analyzed their oxidative modifications by nano LC-MS/MS spectrometry (Supplementary Figure S5). Recombinant GSNOR was purified under reducing condition, than was oxidized with 500 µM H_2O_2 (equivalent to 100:1 molar ratio of H_2O_2 to GSNOR protein) and with 5 mM H_2O_2. Afterward, free cysteine residues were blocked with iodoacetamide and reversibly modified cysteine residues were reduced with DTT and labeled with *S*-methyl-methanethiosulfonate (MTHIO). After chymotryptic digestion, the cysteine containing peptides were analyzed for their modifications by nano-LC-MS/MS. Peptides containing Cys94, Cys99, Cys102, and Cys105 could not be detected at all. All other cysteine residues could be identified in its reduced form and the average amount of free thiols (−SH) was 90% in the water treated samples (**Figure 6A**). Increasing concentrations of H_2O_2 increased the amount of oxidized cysteine residues. We identified both reversibly (MTHIO-labeled) and irreversibly (sulfinic or sulfonic acids, SO_xH) modified cysteine residues (Supplementary Figure S5). According to the increasing concentration of H_2O_2 the average frequency of MTHIO-labeled peptides increased from 17% to around 40% and the SO_xH-modified cysteines from 8 to 55% (**Figure 6A**). Three cysteine residues (Cys47, Cys177, and Cys271) are located in the substrate-binding site of GSNOR highlighted in the three-dimensional structure of *Arabidopsis* GSNOR (**Figure 6B**). Cys47 and Cys177 are coordinating the catalytic Zn^{2+} together with His69 and water molecule. H_2O_2 treatment increased the amount of oxidative modifications for both Zn^{2+}-coordinating cysteines (**Figure 6C**). Around 35% of Cys47 was already oxidized in water-treated GSNOR and the abundance of both MTHIO and SO_xH-modification increased significantly in the presence of 0.5 and 5 mM H_2O_2 (66 and 80%, respectively). This indicates that Cys47 is very sensitive for oxidation. The other Zn^{2+}-coordinating cysteine Cys177 also showed increased reversible oxidation up to 55% after 5 mM H_2O_2 treatment. Interestingly, Cys271, which is located in the NAD^+ cofactor-binding site, was mainly found in its reduced form independent of the treatment. To analyze the importance of these three cysteines, we have generated GSNOR mutants, where these cysteine residues were exchanged by serine resulting $GSNOR^{C47S}$, $GSNOR^{C177S}$, and $GSNOR^{C271S}$. $GSNOR^{C47S}$ and $GSNOR^{C177S}$ showed drastically reduced specific activity (100-fold less and 50-fold less, respectively) compared to WT GSNOR (**Figure 6D**) demonstrating the importance of these two cysteines. In contrast, the mutation of Cys271 resulted in twofold increase of the specific activity.

H_2O_2-Induced Zn^{2+} Release of GSNOR

S-nitrosoglutathione-reductase contains two Zn^{2+} per subunit; one is located in the active center of protein called catalytic

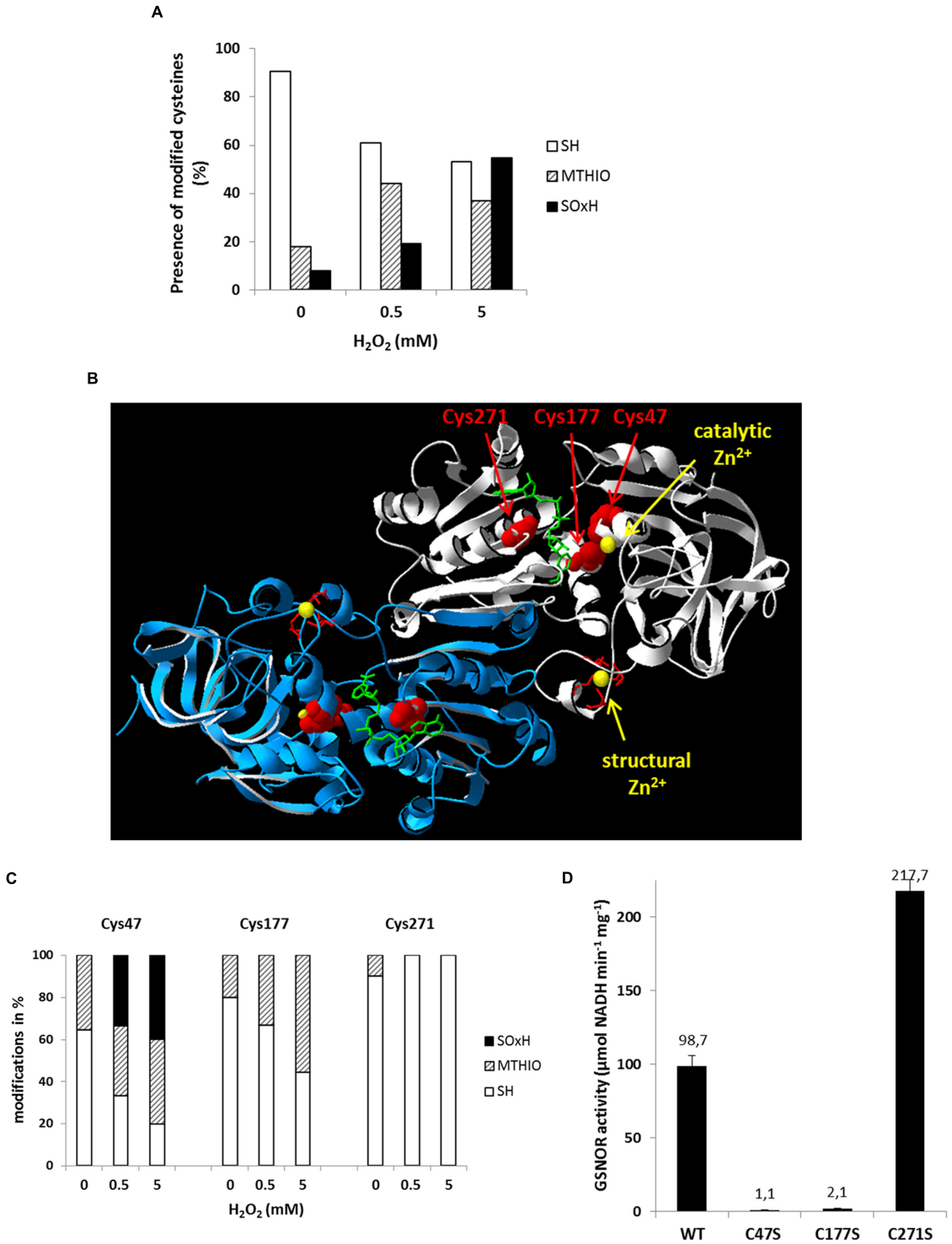

FIGURE 6 | Oxidative modifications of multiple cysteine residues of GSNOR correlate to reduced activity. (A) nano LC-MS/MS analysis of cysteine residues of recombinant GSNOR oxidized with 0.5 and 5 mM H_2O_2. The portion of different modification is represented as the mean percentage of all detected peptides (modifications of individual cysteines are shown in Supplementary Figure S1). SH represents free cysteines, MTHIO-labeling shows reversibly modifications, and SOxH represents irreversibly oxidative modifications. **(B)** Percentage distribution of different modifications (SH, MTHIO, and SOxH) of Cys47, Cys177, and Cys271 residues of oxidized GSNOR by nano LC-MS/MS. **(C)** 3D structure of *Arabidopsis* GSNOR (PDB code: 3UKO) as a homodimer (two subunit is labeled by white and blue). Cysteine residues in the substrate-binding site are highlighted in red. The bound NAD^+ cofactor is shown by green sticks. The catalytic and structural Zn^{2+} is labeled by yellow. **(D)** Specific enzyme activity was determined of WT GSNOR and cysteine mutants $GSNOR^{C47S}$, $GSNOR^{C177S}$, and $GSNOR^{C271S}$. The mean values with SD of three determinations are presented in the graph.

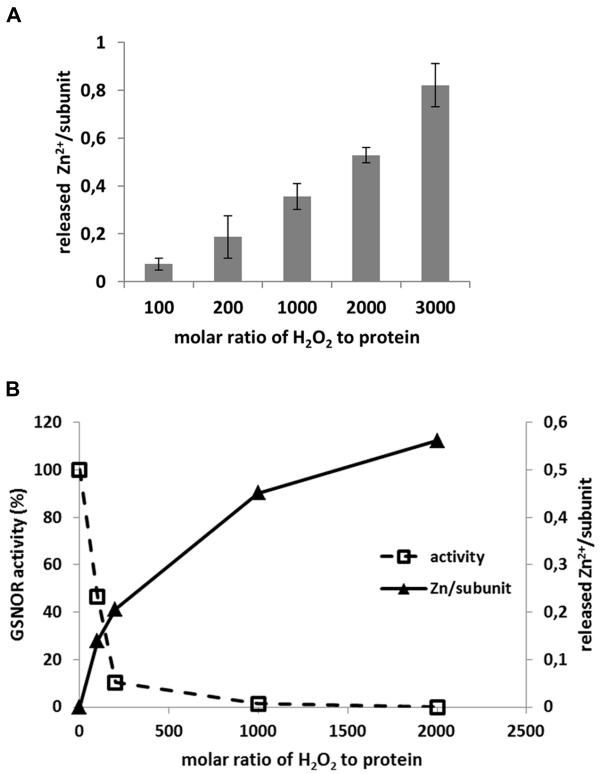

FIGURE 7 | H_2O_2-induced Zn^{2+} release of GSNOR protein. **(A)** Determination of Zn^{2+} release of recombinant GSNOR (50–100 μg) incubated for 1 h with increasing molar excess of H_2O_2 as indicated. The measured Zn^{2+} was first corrected by untreated GSNOR sample (background correction) and then calculated to one subunit of GSNOR. **(B)** Representative experiment for parallel measurement of GSNOR activity and Zn^{2+} release using the same oxidized sample as indicated. GSNOR activity is presented as percentage of untreated protein. The released Zn^{2+} is calculated to one subunit of GSNOR.

Zn^{2+}, the other one is structural Zn^{2+}. The catalytic Zn^{2+} is coordinated by Cys47 and Cys177 and both are sensitive for oxidation (**Figure 6C**). Therefore, we tested whether oxidative inhibition of enzyme correlates with Zn^{2+}-release. Treatment of recombinant GSNOR protein with H_2O_2 resulted in Zn^{2+}-release in a H_2O_2 concentration-dependent manner (**Figure 7A**). We observed a maximum release of 0.82 Zn^{2+} (±0.16) calculated for one subunit of the dimer with the highest molar excess of H_2O_2 (3000-fold excess corresponded to 20 mM H_2O_2). The activity measurement of the H_2O_2-treated GSNOR showed that the enzyme was completely inhibited by 1000-fold molar excess of H_2O_2 (**Figure 7B**). This corresponds to a release of 0.4 Zn^{2+}/subunit (**Figure 7B**) suggesting a correlation between activity loss and Zn^{2+}-release. We could not measure higher Zn^{2+}-release than 0.98 Zn^{2+}/subunit indicating that the second Zn^{2+} atom (the structural one) is probably not affected by oxidation. Interestingly, excess of external Zn^{2+} prevented H_2O_2-caused inhibition of GSNOR activity (Supplementary Figure S6), further confirming, that loss of Zn^{2+} results in loss of GSNOR activity.

DISCUSSION

The term oxidative stress describes the temporary imbalance of the cellular redox homeostasis due to enhanced accumulation of ROS triggering both signaling events and damaging processes (Foyer and Noctor, 2013). A change in ROS homeostasis and the associated shift in the redox state are induced primarily by external environmental influences during various abiotic and biotic stress treatments summarized in Mittler (2002). The major posttranslational protein modifications arising from interaction with ROS are oxidation of sulfur-containing residues (cysteine, methionine) and aromatic residues (tyrosine, tryptophan), carbonylation reactions and formation of disulfide bridges (Rinalducci et al., 2008; Waszczak et al., 2015). Besides ROS, reactive nitrogen species are very important signaling molecules in plants involved in biotic and abiotic stress responses. GSNOR activity controls intracellular levels of GSNO and *S*-nitrosylated proteins and the physiological importance of GSNOR in fine-tuning NO/SNO levels during many stress responses and in plant growth and development is well described. To investigate the effect of oxidative modifications on individual proteins is crucial in understanding the signaling responses under different stress conditions. We demonstrate that GSNOR activity is inhibited by H_2O_2 *in vitro* or paraquat *in vivo* (**Figures 1A** and **4A**), providing a new evidence for crosstalk between ROS and NO signaling. H_2O_2-dependent inhibition of GSNOR activity was also observed in *Baccaurea ramiflora* (Burmese grape) investigating chilling stress (Bai et al., 2012). Moreover, alcohol dehydrogenase 1 (YADH1) from *Saccharomyces cerevisiae* was also inhibited by H_2O_2 *in vitro* due to oxidative modifications of specific cysteine residues (Men and Wang, 2007). This enzyme is structurally related to GSNOR (class III alcohol dehydrogenase). After H_2O_2-treatment, Cys43 and Cys153 of YADH1 were oxidized and three disulfide bonds (Cys43–Cys153, Cys103–Cys111, Cys276–Cys277) were detected (Men and Wang, 2007). Cys43 and Cys153 of YADH1 correspond to *Arabidopsis* GSNOR Cys47 and Cys177 and they are conserved residues in class III alcohol dehydrogenases coordinating the catalytic Zn^{2+}. In correlation with YADH1, we also observed oxidative modifications of these two residues (reversible and/or SO_2H and SO_3H) by MS analyses of H_2O_2-treated *Arabidopsis* GSNOR (**Figure 6**). Moreover, our results show that nearly all detected cysteine residues were accessible to oxidative modification in a dose-dependent manner (Supplementary Figure S5). In contrast to YADH1, we could not detect a disulphide formation of *Arabidopsis* GSNOR by a shift on non-reducing SDS-PAGE (Supplementary Figure S4) and also not by MS (data not shown). Substitution of Cys47 and Cys177 to serine residues resulted in a loss of GSNOR activity (**Figure 6D**) indicating the importance of a Zn^{2+}-thiolate catalytic center. Early biochemical studies in mammals showed evidence that oxidation of cysteines of zinc-finger transcription factors can abolish DNA binding and transcriptional functions (Webster et al., 2001). Superoxide-induced Zn^{2+} release has also been demonstrated in the zinc finger motif of protein kinase C (Knapp and Klann, 2000). Furthermore, investigation of different oxidants on the oxidative Zn^{2+} release in YADH1 revealed an inverse correlation between

alcohol dehydrogenase activity and the released Zn^{2+} (Daiber et al., 2002). The strongest oxidant was peroxynitrite leading to release of one zinc atom/subunit of YADH1, following H_2O_2 and the less effective was NO. Oxidation of recombinant *Arabidopsis* GSNOR by H_2O_2 also resulted in a Zn^{2+} release (**Figure 7**). Similarly, *Arabidopsis* GSNOR contains two Zn^{2+} per subunit, however, we observed the release of only one Zn^{2+}/subunit at the highest excess of H_2O_2 (at molar excess of 3000). Since the Zn^{2+}-release has been accompanied by loss of activity we assumed that most likely Zn^{2+} from the active center of the protein is released. Moreover, Cys47, which is involved in coordinating the catalytic Zn^{2+}, is very sensitive to oxidation (**Figure 6C**). The second Zn^{2+} (structural Zn^{2+}) is coordinated by four cysteine residues (Cys94, Cys99, Cys102, and Cys105) and is not involved in the enzymatic activity of GSNOR. Interestingly, *S*-nitrosation of conserved non-zinc coordinating cysteines (Cys10, Cys271, and Cys370) were reported very recently and this modification was shown to cause a catalytic inhibition of *Arabidopsis* GSNOR (Guerra et al., 2016).

To analyze the ROS-induced inhibition of *Arabidopsis* GSNOR *in vivo*, the bipyridium herbicide paraquat was used as a ROS-inducing agent (Vaughn and Duke, 1983). The redox cycling of paraquat with molecular oxygen produces superoxide radical, which is then mainly dismutated by superoxide dismutase (SOD) to H_2O_2 (Bus and Gibson, 1984). However, in the presence of NO, peroxynitrite is formed from the reaction between O_2^- and NO, which is approximately six-times faster than the dismutation by SOD (Pacher et al., 2007). $ONOO^-$ is a powerful oxidant and nitrosating compound in the cellular environment modifying amino acids, nucleic acids, low and high molecular weight thiols and phospholipids. Paraquat reversibly inhibits GSNOR activity (**Figure 1A**) resulting in enhanced levels of SNOs (**Figure 2A**). Plants that lack GSNOR activity are more tolerant toward paraquat than WT plants, which develop cell death phenotype germinated on 0.5–1 μM paraquat-containing media (**Figures 1C,D**) (Chen et al., 2009) suggesting an activated resistance mechanisms in *gsnor* plants. Enhanced levels of cellular SNOs in *gsnor* in comparison to WT plants (**Figure 2A**) might be responsible for the observed tolerance against paraquat-induced oxidative stress. In correlation, SNO levels increased more than twofold in WT plants after paraquat treatment (**Figure 2A**) providing evidence for ROS-induced inactivation of GSNOR *in vivo*. Co-treatment of NO donor sodium nitroprusside and paraquat during germination of WT plants resulted in increased resistance to paraquat supports this hypothesis (Chen et al., 2009). A protective effect of NO against paraquat-induced oxidative stress was also described in potato and rice after incubation with NO-releasing compounds, however, the exact mechanism is not provided (Beligni and Lamattina, 1999; Hung et al., 2002). Normally, higher SNO/NO levels in *gsnor* plants should increase the production of $ONOO^-$ during paraquat treatment. In contrast, we observed a reduced tyrosine nitration level as a marker for $ONOO^-$ production in the paraquat-treated *gsnor* plant (Supplementary Figure S1B). This result indicates that either the production or the turnover of $ONOO^-$ is affected by excess NO/SNO. It was demonstrated in soybean cells that $ONOO^-$ is not a determining factor of hypersensitive cell death, but the common action of NO and H_2O_2 (Delledonne et al., 2001). The paraquat-treated *gsnor* mutant accumulates lower amount of H_2O_2 than the WT plant (Supplementary Figure S3) supporting the scavenging function of NO to decrease excess level of ROS species. On one side, peroxynitrite formation can be a mechanism to consume superoxide thereby protecting biomolecules from oxidation and preventing further ROS production (Kanner et al., 1991; Wink et al., 2003). On the other side, $ONOO^-$ can inhibit several isoforms of SOD (Holzmeister et al., 2015) resulting in less H_2O_2 production. Besides the scavenging function of NO, the plant cell can overcome elevated ROS levels by activating the antioxidant system. GSH is one of the major low molecular weight thiol, which reacts rapidly to changing stress situations and is crucial to maintain cellular redox balance. Both, loss of GSNOR function and fumigation with NO enhanced GSH level (**Figures 3A,C**) assuming that NO/SNO is able to stimulate the GSH biosynthesis pathway. These measurements coincide with previous reports demonstrating a higher amount of GSH in roots of *Medicago truncatula* (Innocenti et al., 2007) and maize leaves (Mello et al., 2012) after GSNO and SNP treatment, respectively. In both cases, an enhanced expression of the γ-glutamylcysteine synthetase and GSH synthetase gene was detectable suggesting a NO-dependent transcriptional regulation of GSH production. Together with a twofold higher glutathione content in *gsnor* plants, we measured increased glutathione reductase activity (**Figure 3B**), which is responsible for the recovery of GSH and thus the maintenance of the redox homeostasis. Assuming that the GR activity is involved in GSH regeneration, the *gsnor* plants would therefore be able to provide more reducing equivalents needed for the stress response (**Figure 3A**). Moreover, increased conjugase activity of GST was measured in *gsnor* plants with and without paraquat treatment (**Figure 3B**). Enhanced GST activity could be observed in response to different abiotic and biotic stimuli and their activity is important to protect plants against oxidative damage (Sappl et al., 2009). The induction of the antioxidant system in *gsnor* plants was further demonstrated by a transcript profile analysis. Comparison of WT and *gsnor* plants under normal growth condition revealed an enhanced expression of genes involved in antioxidant processes in *gsnor* plants (**Table 1**). The up-regulated genes of peroxidases or GSTs are markers for oxidative stress and/or H_2O_2 signaling (Vanderauwera et al., 2005; Queval et al., 2012). However, little is known about the exact physiological function of these enzymes during oxidative stress. Interestingly, several members of the Tau class GSTs are also upregulated during paraquat and H_2O_2 treatment of *Arabidopsis* seedlings (Genevestigator At-413 and Genevestigator At-185). Moreover, using a yeast two-hybrid approach a tomato cDNA library was screened for "proteins" protecting yeast from prooxidant–induced cell death. In this screen five homologous Tau class GSTs were identified concluding that especially this class of GST proteins has a protective function in oxidative stress response (Kilili et al., 2004). The fact that the expression of 13 members of the Tau subfamily of GSTs is upregulated in

gsnor plants in comparison to WT plants suggests that these genes are regulated by SNO/NO and are important for protection against oxidative stress. Although peroxisomal catalases and the ascorbate-glutathione pathway play a primarily role in the metabolism of H_2O_2 (Mhamdi et al., 2010; Noctor et al., 2012), we did not observed any changes in the expression of catalases or genes related to the ascorbate-glutathione-dependent pathway like APX or dehydroascorbate reductases. However, several other classes of antioxidative peroxidases exist that can reduce H_2O_2 and/or organic peroxides. These include thioredoxin-, or glutathione-peroxidases, and glutathione-*S*-transferases (Dietz, 2003; Dixon et al., 2009). Based on our microarray data an alternative pathway involving SNO/NO-induced thioredoxin- and/or glutathione-dependent peroxidases might be present and result in activation of the antioxidative system.

Furthermore, a thiol protective role of *S*-nitrosylation has been reported in animals (Evangelista et al., 2013). Formation of higher order irreversible oxidative modifications, such as sulfinic and sulfonic acids were prevented by *S*-nitrosylation. Recent paper has provided evidence that *S*-nitrosylation of *Arabidopsis* APX1 enhances its activity to scavenge H_2O_2 and to increase resistance to oxidative stress (Yang et al., 2015). *S*-Nitrosylation of pea APX also enhanced its enzyme activity in saline stress (Begara-Morales et al., 2014). Moreover, the activity of NADPH oxidase is inhibited by *S*-nitrosylation, resulting in the reduction in ROS biosynthesis during immune responses (Yun et al., 2011). Interestingly, activity of *Arabidopsis* GSNOR is inhibited by *S*-nitrosylation demonstrating that SNOs control its own scavenging by modulating GSNOR activity (Frungillo et al., 2014; Guerra et al., 2016).

In sum, we demonstrated that GSNOR activity can be inhibited *in vitro* by H_2O_2, as well as *in vivo* by paraquat, which is accompanied by a significant change in NO homeostasis. The observed increase in cellular SNOs consequently leads to induction of NO-dependent signaling mechanisms, resulting in GSH accumulation, enhanced activity of GSH-related enzymes and finally in a protection against oxidative stress. All these findings substantiate the physiological importance of GSNOR in fine-tuning the levels of NO/SNO during plant growth and development and also in many stress response reactions.

AUTHOR CONTRIBUTIONS

IK, CH, and CL designed research. IK, CH, MW, AG, GR, TF, GK, and EL performed research. IK, CH, MW, AG, TF, RH, GA, JD, and CL analyzed data. IK, CH, and CL wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01669/full#supplementary-material

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ROS Production and Scavenging under Anoxia and Re-Oxygenation in *Arabidopsis* Cells: A Balance between Redox Signaling and Impairment

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Plants can frequently experience low oxygen concentrations due to environmental factors such as flooding or waterlogging. It has been reported that both anoxia and the transition from anoxia to re-oxygenation determine a strong imbalance in the cellular redox state involving the production of reactive oxygen species (ROS) and nitric oxide (NO). Plant cell cultures can be a suitable system to study the response to oxygen deprivation stress since a close control of physicochemical parameters is available when using bioreactors. For this purpose, *Arabidopsis* cell suspension cultures grown in a stirred bioreactor were subjected to a severe anoxic stress and analyzed during anoxia and re-oxygenation for alteration in ROS and NO as well as in antioxidant enzymes and metabolites. The results obtained by confocal microscopy showed the dramatic increase of ROS, H_2O_2, and NO during the anoxic shock. All the ascorbate-glutathione related parameters were altered during anoxia but restored during re-oxygenation. Anoxia also induced a slight but significant increase of α-tocopherol levels measured at the end of the treatment. Overall, the evaluation of cell defenses during anoxia and re-oxygenation in *Arabidopsis* cell cultures revealed that the immediate response involving the overproduction of reactive species activated the antioxidant machinery including ascorbate-glutathione system, α-tocopherol and the ROS-scavenging enzymes ascorbate peroxidase, catalase, and peroxidase making cells able to counteract the stress toward cell survival.

Keywords: anoxic stress, antioxidant defenses, bioreactor, hydrogen peroxide, nitric oxide, reactive oxygen species

Abbreviations: APX, ascorbate peroxidase; ASC, ascorbate; carboxy-H_2DCFDA, 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; CAT, catalase; DAF-2 DA, 4,5-diamino-fluorescein diacetate; DHAR, dehydroascorbate oxidoreductase; DHR, dihydrorhodamine 123; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDHAR, monodehydroascorbate reductase; NO, nitric oxide; POD, peroxidase; ROS, reactive oxygen species.

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INTRODUCTION

Plants being aerobic organisms need oxygen as an essential substrate for energy production. However, oxygen can fall to low concentrations in many plant tissues because of environmental factors such as flooding or waterlogging which reduce the external oxygen concentration. As well, the poor distribution efficiency for oxygen through plant organs or high rates of cellular metabolism in dividing meristem cells can induce a severe drop of plant internal oxygen concentrations. Oxygen deprivation stress in plant cells includes three different states characterized by different oxygen concentrations: hypoxia, anoxia, and re-oxygenation. The responses to oxygen deprivation include various alterations of plant metabolism aimed at plant survival such as energy preservation, reduction of respiration, and induction of fermentation which occurs when oxygen levels are close to zero (Geigenberger, 2003). Microarray analyses revealed several genes highly responsive to oxygen deficiency in different plant species (Mustroph et al., 2010). Recently, an oxygen sensing mechanism involving specific transcription factors, able to trigger the plant molecular response to hypoxia was identified (Licausi et al., 2011; Sasidharan and Mustroph, 2011).

In addition, a mechanism of ROS signaling, involving the production of hydrogen peroxide and acting under oxygen deprivation, was found to be linked to oxygen sensing (Gonzali et al., 2015).

Anatomical and morphological changes such as aerenchyma formation also occur in several plant species to cope with frequent flooding. This adaptation, aimed at improving gas exchange, is mediated by ROS, in particular hydrogen peroxide (Steffens et al., 2011).

It has been reported that in plants during the transition to hypoxia/anoxia from normoxic conditions or during re-oxygenation, ROS and NO can be excessively produced. Paradoxically plant responses to oxygen deprivation appear to include the production of hydrogen peroxide or other ROS (Bailey-Serres and Chang, 2005; Blokhina and Fagerstedt, 2010; Irfan et al., 2010). In these conditions, an impairment of redox balance occurs that requires the activation of defense pathways involving redox metabolites and related enzymes responsible to counteract ROS production and to recover redox homeostasis. The plant redox defense system is represented by several molecules, both hydrophilic and lipophilic, exerting their effects in different cell compartments such as ASC, glutathione and tocopherols. In addition, enzymes able to directly eliminate ROS excesses can be activated: superoxide dismutases, CATs, and PODs using different electron donor as well as enzymes responsible for the recycling of redox pairs, in particular ASC and glutathione.

Several studies have been carried out on the redox balance of plant tissues under oxygen deprivation stress, however, variable responses have been reported for different plant species and different experimental conditions; in particular, the levels of antioxidant molecules and the activity of antioxidant regenerating enzymes did not show equal trends (Blokhina et al., 2003). The resistance of a plant species/variety to anoxic stress, the physiological conditions of the exposed plant, as well as stress intensity are factors affecting plant resilience.

Establishing experimental conditions, which make plant cells uniformly exposed to a controlled oxygen supply, is not simple to achieve. Cultivating plant cells in a bioreactor system makes it possible to control physicochemical parameters to be homogeneous throughout the culture. Furthermore, the heterogeneity of oxygen availability often occurring in different cells or tissues in the same organ is avoided in suspension cells. Therefore, the use of bioreactors can be a suitable system for studying plant cell response to oxygen deprivation. In a previous work, *Arabidopsis* cell cultures subjected to hypoxia (static flasks) or anoxia (bioreactor) revealed a defense response involving an increase of the levels of H_2O_2 in the medium and the antioxidant metabolite α-tocopherol (Nisi et al., 2010). To shed more light on this response, *Arabidopsis* cell suspension cultures grown in a stirred bioreactor were subjected to a severe anoxic stress and analyzed during anoxia and re-oxygenation for the alteration in ROS, H_2O_2, and NO as well as in antioxidant enzymes and metabolites.

MATERIALS AND METHODS

Cell Cultures

Cell suspension cultures of *Arabidopsis thaliana* L., Heynh., ecotype Landsberg were maintained in MS (Murashige and Skoog, 1962) medium supplemented with 30 g l^{-1} sucrose, 0.5 mg l^{-1} NAA (naphthaleneacetic acid), 0.05 mg l^{-1} Kinetin. Cell suspensions were subcultured in 500 ml flasks at 15-day intervals by inoculating 2 ml of packed cell volume in 50 ml of fresh medium.

MS medium (3.150 l) containing 30 g l^{-1} sucrose, 0.5 mg l^{-1} NAA, 0.05 mg l^{-1} Kinetin was inoculated with 350 ml of 14-day-old shake flask suspension in a 5.0 l stirred bioreactor (BioFlo 110, New Brunswick Scientific, Edison, NJ, USA). Cultivation was performed at 25°C, pH 5.6 and 80 rpm agitation speed, under continuous fluorescent white light (50 μmol photons m^{-2} s^{-1}). Before the anoxic stress, cells were cultivated under 20% dissolved oxygen (DO) of air saturation, automatically obtained by the gas mix controller (New Brunswick Scientific, Edison, NJ, USA).

Experimental Design

Arabidopsis cells grown in a stirred bioreactor for 8 days under 20% DO were subjected to anoxia for 4 h, by stopping aeration and fluxing with nitrogen into the vessel (0.01% DO). During the whole period of treatment, the bioreactor was maintained in the dark. Thereafter they were re-oxygenated by restoring the previous aeration conditions for 20 h. All the analyses were performed on samples taken at different times: T0, normoxia; T1 and T2, anoxia for 2 and 4 h, respectively; T3 and T4, 2 and 20 h after re-oxygenation, respectively. T1 and T2 samples were collected under nitrogen flux and immediately used for avoiding re-oxygenation.

Cell Viability

Arabidopsis cell suspension cultures were stained with the Evans Blue dye and cell death was determined by spectrophotometric analysis according to Carimi et al. (2003). For each time, three independent experiments were performed with each assay done in triplicate. Dead cells were also analyzed by light microscopy according to de Pinto et al. (1999). For each treatment, 500 cells were examined.

Ascorbate and Glutathione Analyses

For ASC and glutathione determination, 0.3 g cells were homogenized with cold 5% metaphosphoric acid at 4°C at 1:3 ratio (w/v) in order to obtain a deproteinized extracts. After centrifugation at 20000 g for 20 min, the supernatants were collected and used for ASC and GSH analysis according to Paradiso et al. (2006).

Extraction and Analysis of Tocopherols

Extraction and analysis of tocopherols were carried out as previously described (Caretto et al., 2002). Briefly, the method consisted of an alkaline hydrolysis (potassium hydroxide 60%) followed by extraction with *n*-hexane-ethyl acetate (9:1). Chromatography separation was performed by using a Beckman HPLC Analytical System. A RPC18 Beckman Ultrasphere column was used with methanol (98%) as the mobile phase. Two programmable detectors, an ultraviolet-visible spectrophotometer (set at λ: 290 nm) and a spectrofluorimeter (λ excitation: 289 nm; λ emission: 325 nm) were connected in series to determine tocopherols. The tocopherol content was calculated by means of standard calibration curves. Each experiment was carried out with at least three replicates.

ROS, NO, and H_2O_2 Detection by Confocal Laser Scanning Microscopy (CLSM)

A confocal laser scanning microscope system, CLSM (Zeiss LSM Pascal, Carl Zeiss Inc., Germany), equipped with He-Ne and Ar lasers and coupled to Axiovert 200 inverted microscope (Zeiss, Germany) was used to detect ROS, NO, and H_2O_2 in anoxia-treated and untreated *Arabidopsis* cells by using specific fluorescent probes.

Reactive oxygen species were detected by using 25 μM carboxy-H_2DCFDA in DMSO, from Image-iT^{TM} LIVE Green ROS Detection Kit (Molecular Probes, Invitrogen, Ltd., Paisley PA4 9RF, UK) following the manufacturer instructions. Two hundred microliters of anoxia-treated cell cultures, at different times, and the relative controls, were incubated for 30 min at 25°C, in darkness, with 25 μM carboxy-H_2DCFDA probe. Then cell suspensions were centrifuged at 2000 × *g*, washed twice with 1 ml of fresh medium and finely suspended in 200 μl of medium. Cells were immediately observed by CLSM.

Hydrogen peroxide (H_2O_2) presence was detected by DHR probe (Life Technologies, Carlsbad, CA, USA). Two hundred and fifty microliters of anoxia-treated and control cells were incubated 10 min with 1 μl of DHR, in the darkness. The medium was then removed and the cells were washed twice with fresh medium and immediately imaged by CLSM. The specificity of the staining for H_2O_2 was tested in a parallel experiment by adding 30 U ml^{-1} of CAT before the addition of DHR.

For NO detection a 250 μl of cell culture sample, were incubated for 10 min at 25°C, in darkness, with 1 μl of DAF-2 DA (Calbiochem-Novabiochem) diluted in medium, then cells were centrifuged at 2000 × *g*, washed twice in fresh medium and immediately imaged by CLSM.

Carboxy-H_2DCFDA, DAF-2 DA, and DHR probes were excited with the 488 nm line, and filtered through a 505–530 nm band pass filter, fluorescence was displayed as green false color. The second channel collecting emission beyond 650 nm by a long band-pass filter (excited at 488 nm laser line) was used to collect the autofluorescence of chlorophylls and the highly excited R123, and was displayed as red false-color. Background staining, routinely negligible, was controlled with unstained cells, which showed only the red autofluorescence of chloroplasts (data not shown). Confocal images were recorded using Plan-Neofluar 20×/0.5 and Plan-Neofluar 40×/0.75 objectives. A minimum of nine randomly taken fields, per treated and not treated cell cultures, was recorded. LSM Pascal 5 software was used to record and match confocal images.

Enzyme Assays

Cells were ground in liquid nitrogen with a mortar and pestle. Five volumes of a buffer containing 50 mM Tris-HCl (pH 7.5), 0.05% (w/v) cysteine, 0.1% (w/v) BSA, were added just as the last trace of liquid N_2 disappeared. The thawed mixture was then ground and centrifuged at 20000 g for 15 min. The supernatant was used for spectrophotometric analysis.

Cytosolic APX (L-ascorbate: hydrogen peroxide oxido-reductase, EC 1.11.1.11) activity was measured according to Locato et al. (2009) by following the H_2O_2-dependent oxidation of ASC at 290 nm in a reaction mixture containing 0.1 M Tris-acetate buffer, pH 6.4, 350 μM ASC, 170 μM H_2O_2, 50–100 μg protein. The non-enzymatic H_2O_2-dependent oxidation of ASC, as well as the oxidation of ASC, not dependent upon H_2O_2 addition, was subtracted.

Dehydroascorbate oxido-reductase (glutathione: DHAR, EC 1.8.5.1), MDHAR (NADH: monodehydroascorbate radical oxido-reductase, EC 1.6.5.4), and CAT (hydrogen peroxide: hydrogen peroxide oxido-reductase, EC 1.11.1.11) were assayed according to Paradiso et al. (2012). Peroxidase activity (POD EC 1.11.1.7) was measured following the oxidation of 3,3′,5,5′-Tetramethylbenzidine (TMB) at 652 nm ($\varepsilon = 26{,}9$ mM^{-1} cm^{-1}), according to Ferrer et al. (1990).

Protein content was determined according to Bradford (1976) using bovine serum albumin as standard. All enzyme activities were measured using a Beckman (Fullerton, CA, USA) DU 7000 spectrophotometer.

Total RNA Extraction and Semi-Quantitative RT-PCR

Total RNA was isolated from *Arabidopsis* cells using the RNeasy plant minikit (QUIAGEN S.p.A., Milan, Italy) according to the supplier's recommendations. Residual DNA was removed from

the RNA samples using a DNA-*free* kit (AMBION, Inc., Austin, TX, USA). Synthesis of cDNA was performed from 2 μg total RNA with 10 μM random primers (AMERSHAM Biosciences Europe GMBH, Milan, Italy), utilizing an Omniscript Reverse Transcriptase kit (QUIAGEN S.p.A., Milan, Italy) according to the supplier's recommendations. PCR reactions were performed with specific primers for APX1 (X59600, 5′-GGACGATGCC ACAAGGATAG-3′ and 5′-GGTTGCGATTTGAACACAT-3′); APX2 (X98275, 5′-ATTGCCGTTAGGCTTCTTGA-3′ and 5′-TACCAACCGACAAGGCTCTT-3′); APX6 (AV555486, 5′-CTG CTGGTGTGCTTCGTTTA-3′ and 5′-TTGAAAAACCATGGA CGTCA-3′) and 18S rRNA (AJ236016, 5′-CATGATAACTCGA CGGATCG-3′ and 5′-GAAGGCCAACGTAATAGGAC-3′). 18S rRNA was used as an internal control in order to normalize each sample for variations in the amount of initial RNA. For semi-quantitative RT-PCR, the cycle number in the linear range was empirically determined. These were analyzed on 1.5% agarose gel containing 0.5 μg mL^{-1} ethidium bromide.

Statistical Analysis

Experiments were repeated five times and a statistical analysis was performed. Data were analyzed by one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Several studies have reported ROS generation in response to oxygen deprivation or re-oxygenation in plants. The ROS, in particular H_2O_2, generated during stress have been implicated as triggers of signaling pathways influencing the expression of nuclear encoded genes which may initiate acclimation processes contributing to stress tolerance (Banti et al., 2013). In this study, a rise in reactive oxygen and nitrogen species during anoxia and the activation of antioxidant system during re-oxygenation is shown in *Arabidopsis* cell suspensions grown in a bioreactor, which allowed the close control of oxygen levels.

Cell Viability

At different time intervals cell samples of *Arabidopsis* cell cultures subjected to anoxia and re-oxygenation were taken and analyzed for cell viability. Since no significant differences were observed between control and treated cells, cell viability was not affected by anoxia followed by re-oxygenation (data not shown).

ROS, H_2O_2, and NO Detection by CLSM

The production of the main reactive species in *Arabidopsis* cell cultures was evaluated during normoxia, anoxia, and re-oxygenation by using fluorescent probes able to detect ROS, H_2O_2, and NO. Only the spotted red fluorescence, due to the autofluorescence of chloroplast chlorophylls was observed in cells incubated in the absence of the fluorescent probes as control (data not shown).

Reactive oxygen species production was detected in *Arabidopsis* cell cultures under different oxygen levels by using carboxy-H_2DCFDA (**Figure 1**). This probe, when deacetylated by intracellular esterases and oxidized by ROS, emits bright green fluorescence. In normoxic conditions (T0), very few cells showed faint fluorescence as defined single spots, likely due to the basal metabolism. During anoxia (T1 and T2), a diffuse green fluorescence was observed in an increasing number of cells indicating that ROS increase affected the whole cell, thus suggesting the occurrence of an oxidative stress, an usual event under different stress conditions. During re-oxygenation (T3 and T4), a reduction of both fluorescent cell number and fluorescence intensity was observed. Twenty hours after the end of anoxia (T4), cells seemed to restore completely the normoxic conditions being ROS detectable as spotted green-fluorescence.

Among ROS, the production of H_2O_2 was reported to increase in several abiotic stress conditions including oxygen

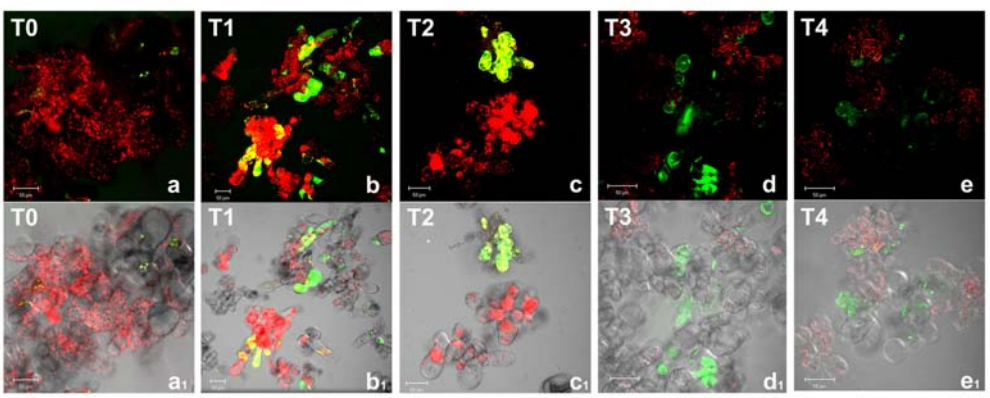

FIGURE 1 | Reactive oxygen species detection by confocal laser scanning microscopy in *Arabidopsis thaliana* cell suspension cultures. Cells were grown in bioreactor in normoxic conditions (T0), subjected to anoxia 2 h (T1) or 4 h (T2) and re-oxygenation 2 h (T3) or 20 h (T4). Merged two-color confocal images **(a–e)** of the green and the red channel show autofluorescence of chloroplasts (red false color) and nonspecific ROS (green false color) detected by carboxy-H_2DCFDA probe. In **(a_1–e_1)** the confocal microscopy images are digitally merged with the transmitted light images for displaying the number of labeled cells in the total cell population. Excitation wavelength 488 nm line, emission was detected at 505–530 nm band pass filter and 650 nm long band-pass filter. Scale bar = 50 μm.

deprivation (Blokhina and Fagerstedt, 2010). This ROS is of particular relevance for its relative stability under different physiological conditions and its capability to cross biological membranes. Indeed, it is well known that alterations in H_2O_2 are part of the redox signaling pathways activating defense responses in all aerobic organisms. H_2O_2 accumulation was then investigated by using 1,2,3-dihydrorhodamine (DHR), a probe which in the presence of H_2O_2 is oxidized to its fluorescent form R123 (Hensley et al., 2003).While DHR passively diffuses across plasma membrane, the charged fluorescent rhodamine-123 cannot cross being trapped in cellular compartments (Kinsey et al., 1987; Henderson and Chappell, 1993). Confocal images (**Figure 2**) show that, in normoxic conditions (T0), only few *Arabidopsis* cells displayed green fluorescence, due to the basal metabolism. Under anoxic conditions, H_2O_2 levels strongly increased. It is worth noting that during anoxia cell boundaries appeared to be particularly involved in H_2O_2 production (T1, T2, and T2m). Several enzymatic systems are responsible for O_2^-/H_2O_2 production at plasma membrane/cell wall interface. Among these, NADPH oxidase and apoplastic superoxide dismutase are well known enzymes participating in ROS overproduction in a plethora of environmental biotic and abiotic stresses, including low oxygen (Pucciariello and Perata, 2016). Specific PODs and amine oxidases also contribute to cell wall O_2^-/H_2O_2 production at least in some cases of stress conditions (Hurkman and Tanaka, 1996; Angelini et al., 2008). At the end of anoxic stress (T2), most *Arabidopsis* cells (about 70–80%) appeared green or yellow-green fluorescent. An increase of H_2O_2 during anoxia is coherent with our previous results indicating a rise for H_2O_2 released in the culture medium during anoxia (Nisi et al., 2010). During re-oxygenation (T3 and T4) H_2O_2 production decreased, only few cells being green fluorescent. The cellular compartment involved in H_2O_2 production also seems to be different being the plasma membrane/cell wall interface probably less relevant and intracellular production more relevant during re-oxygenation than in anoxia. This is consistent with the recovery of mitochondrial electron flow, previously blocked by oxygen deprivation and with a role for H_2O_2 in metabolic signaling within cells.

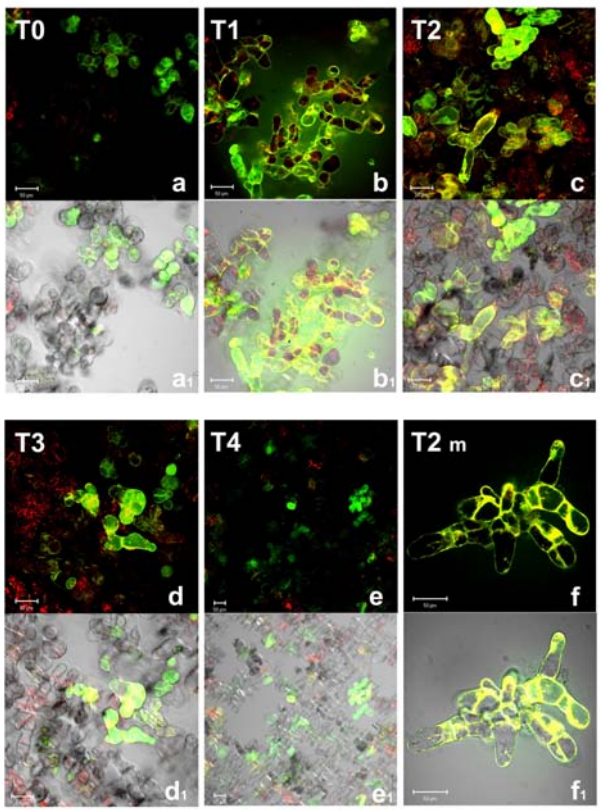

FIGURE 2 | Hydroxyl peroxide (H_2O_2) detection by confocal microscopy in *A. thaliana* cell suspension cultures. Cells were grown in bioreactor in normoxic conditions (T0), subjected to anoxia 2 h (T1) or 4 h (T2) and re-oxygenation 2 h (T3) or 20 h (T4). T2m represents a magnification of T2 cells. Merged two-color confocal images **(a–f)** of the green and the red channel show H_2O_2 (green false color) detected by DHR probe (R123), and autofluorescence of chloroplasts (red false color). In **(a_1–f_1)**, the confocal microscopy images are digitally merged with the transmitted light images for displaying the number of labeled cells in the total cell population. Excitation wavelength 488 nm line, emission was detected at 505–530 nm band pass filter and 650 nm long band-pass filter. Scale bar = 50 μm.

A tight interplay between ROS and reactive nitrogen species has been reported in stress signaling; NO has also been reported to be induced by anoxic conditions (Stöhr and Stremlau, 2006). NO production was then analyzed in *Arabidopsis* cells by CLSM using DAF-2 DA, a probe highly specific for NO (Kojima et al., 1998; Nakatsubo et al., 1998). Because of the short half-life (5–15 s) of NO, the detected fluorescence indicates the amount and the cell localization of NO production at a given time (**Figure 3**).

In normoxia conditions (T0), a basal level of NO production was detected as green fluorescent spots only in few scattered cells (**Figures 3a,a_1**); fluorescence was clearly visible in round shaped endocellular organelles; according to merge picture, almost no production was evident in chloroplasts (**Figures 3f,f_1**). The subcellular site of NO synthesis in plants is still under debate. Among the subcellular compartments suggested to be responsible for such biosynthesis, peroxisomes are probably the best-characterized (Corpas et al., 2013). However, under hypoxic conditions, NO production was also suggested to occur in other compartments such as the cytosol and organelles including mitochondria, which were shown to be a significant source of NO in various organisms including plants (Gupta et al., 2011). During the anoxic stress, a great increase of NO production was observed. It is worth noting that at 2 h anoxia, most cells appeared green fluorescent. At this early stage of anoxia (T1), *Arabidopsis* cells showed a diffuse green fluorescence due to NO within the whole cell volume (**Figures 3b,b_1,g,g_1**). The pre-incubation with the NO scavenger 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide confirmed that green florescence was actually due to NO (data not shown). In T2, ∼60–80% of *Arabidopsis* cells displayed a positive reaction to the probe DAF-2 DA (**Figures 3c,c_1,h,h_1**). During the early recovery period 2 h after re-oxygenation NO was still produced in a relevant number of cells (T3, **Figures 3d,d_1,i,i_1**) even if at lower

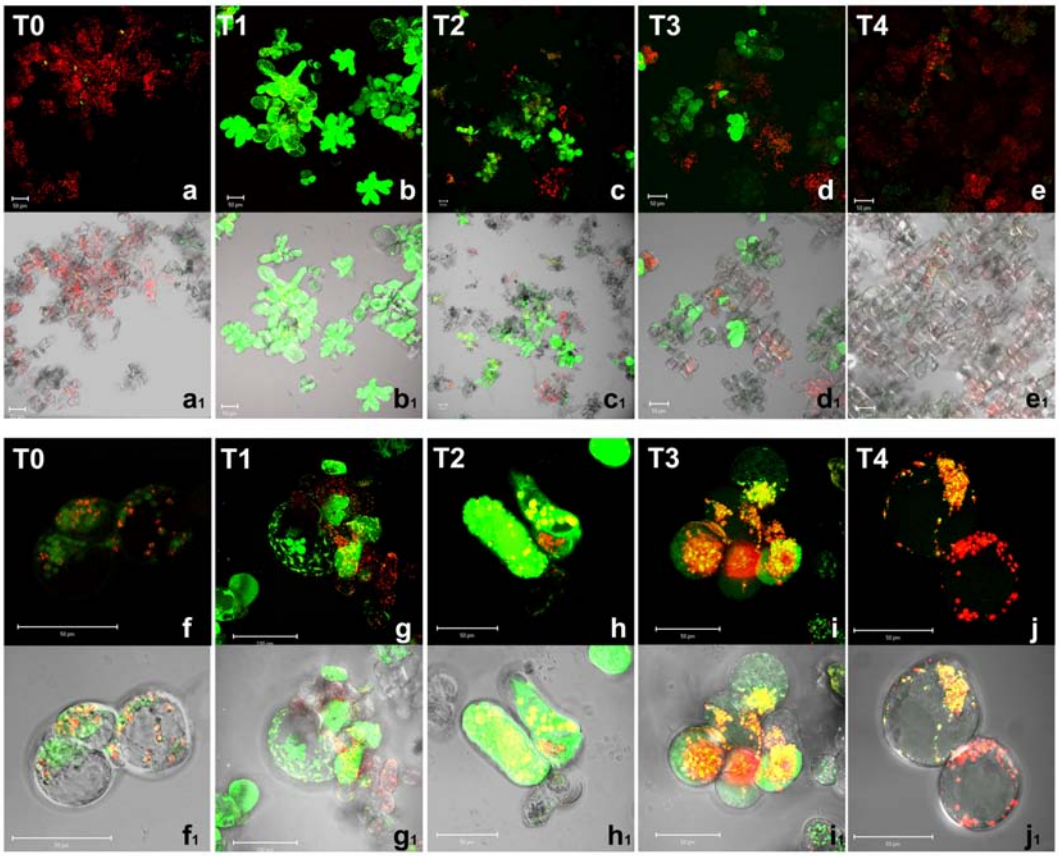

FIGURE 3 | Nitric oxide detection by confocal microscopy in *A. thaliana* cell suspension cultures. Cells were grown in bioreactor in normoxic conditions (T0), subjected to anoxia 2 h (T1) or 4 h (T2) and re-oxygenation 2 h (T3) or 20 h (T4). Merged two-color confocal images **(a–j)** of the green and the red channel show autofluorescence of chloroplasts (red false color) and NO (green false color) detected by DAF-2 DA probe. In **(a_1–j_1)**, the confocal microscopy images are digitally merged with the transmitted light images for displaying the number of labeled cells in the total cell population and the cellular localization of the fluorescent probe. **(f–j)** and **(f_1–j_1)** are at higher magnification. Excitation wavelength 488 nm line, emission was detected at 505–530 nm band pass filter and 650 nm long band-pass filter. Scale bar = 50 μm.

levels compared to T1. After 20 h of re-oxygenation (T4), cells restored the baseline conditions of NO production with very few cells showing faint green fluorescence (**Figures 3e,e**$_1$), which was evident as small dots (**Figures 3j,j**$_1$). Taken together these results indicate that oxygen shortage induced an oxidative burst. ROS and NO production seemed to be particularly high during the 1st hours of anoxia. During re-oxygenation, ROS, and NO were still produced. Under our experimental condition, almost 20 h were required in order to recovery redox homeostasis.

The transient increase of ROS, H_2O_2, and NO in *Arabidopsis* cultured cells during oxygen deprivation was likely able to trigger cell response for allowing the cells to overcome the anoxic stress. Consistently, in spite of such increases, no decrease in cell viability was induced.

ROS Scavenging Systems

In plant cells a complex network of redox metabolites and enzymes guarantees redox homeostasis. This network includes several ROS scavenging enzymes (CAT, PODs) and a network of antioxidant molecules, such as ascorbic acid, glutathione, α-tocopherol, that interact with ROS directly or in reactions carried out by antioxidant enzymes such as APX (Noctor and Foyer, 1998; Apel and Hirt, 2004). In addition, a whole array of enzymes is needed for the regeneration of the active forms of the antioxidant molecules (MDHAR, DHAR, and GR).

The importance of the ASC-glutathione system for the detoxification of hydrogen peroxide has been well characterized in different stress conditions (Locato et al., 2009; de Pinto et al., 2015) but little detailed information is available about its action in anoxic stress.

The behavior of ASC pool during anoxia indicates that no significant alteration occurred when the total ASC plus DHA is considered (**Figure 4A**). In contrast, the redox state (ASC/ASC + DHA) was severely affected by the variations of oxygen concentration. After 2 h of anoxia (T1), a significant increase of the oxidized form (DHA) was observed, resulting in a decrease of the redox state (0.82 ± 0.15 and 0.11 ± 0.042 at T0 and T1, respectively) that remained unchanged up to the end of anoxia (T2). This confirms the strong redox impairment occurring during anoxia. After 2 h of re-oxygenation (T3), however, the

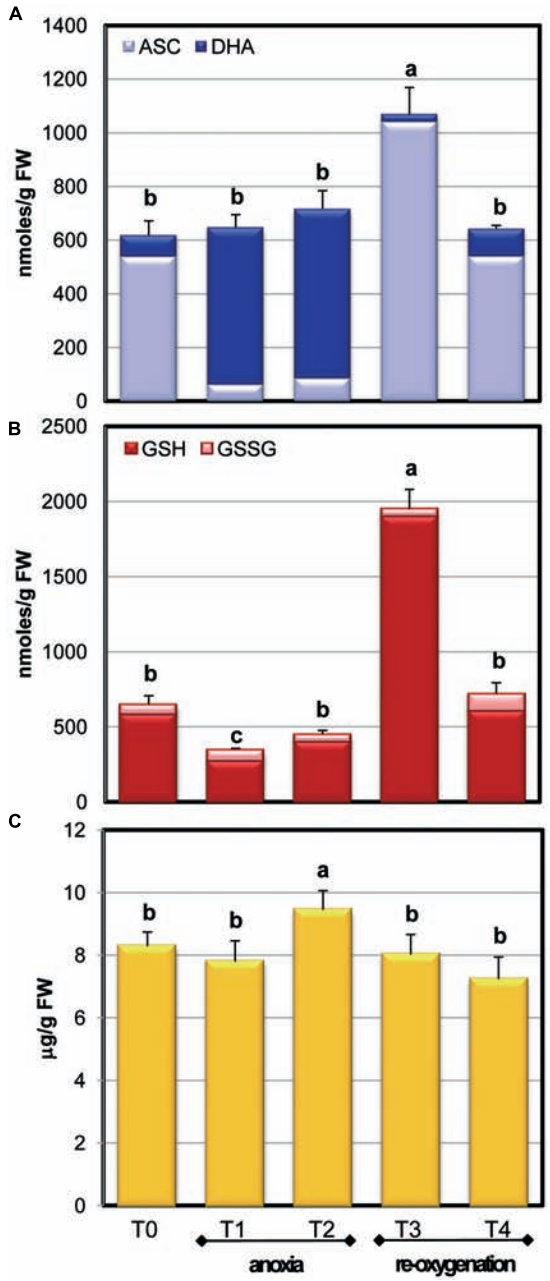

FIGURE 4 | Effects of anoxia and re-oxygenation on ASC pool, GSH pool, and α-tocopherol in *A. thaliana* cell suspension cultures. ASC +DHA **(A)** and GSH+GSSG **(B)** contents are expressed as nmoles/g FW; α-tocopherol **(C)** as µg/g FW. Data represent the means (±SE) of five experiments. Different letters represent values which are statistically different (by one-way Anova test).

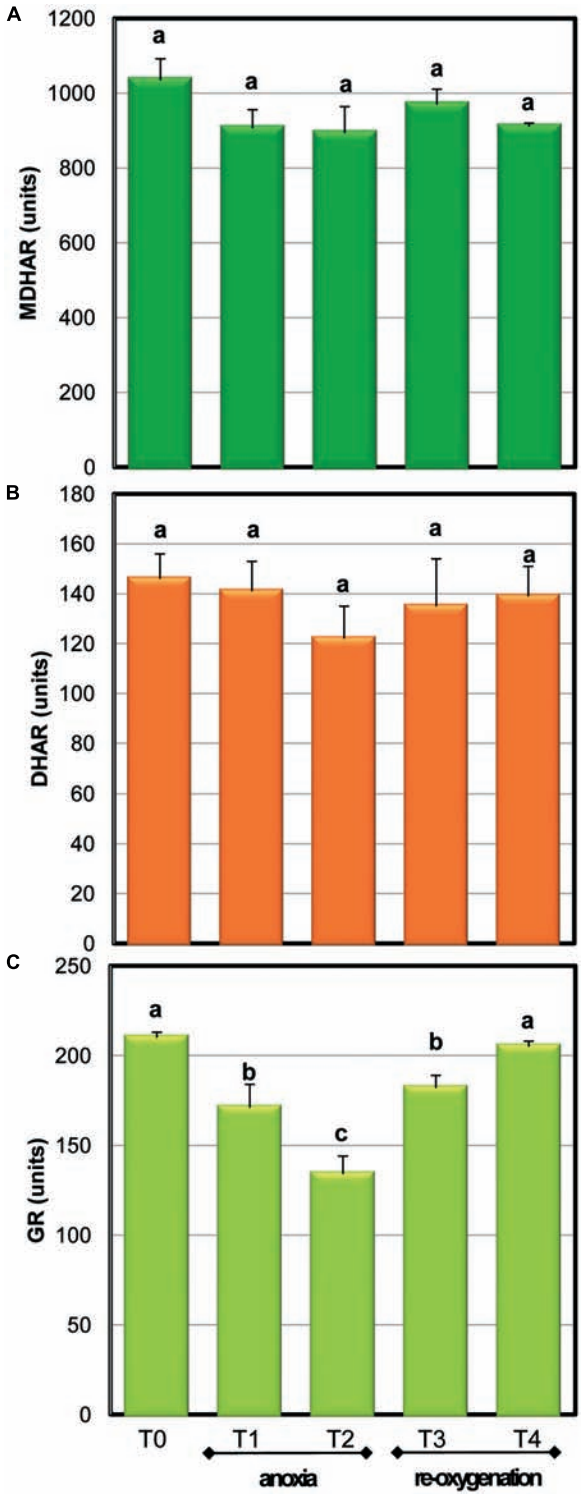

FIGURE 5 | Effects of anoxia and re-oxygenation on ASC-GSH recycling enzymes in *A. thaliana* cell suspension cultures. Activities are expressed in units. MDHAR **(A)**: 1 unit = 1nmol NADH oxidized $min^{-1}mg^{-1}$ prot; DHAR **(B)**: 1 unit = 1nmol DHA reduced $min^{-1}mg^{-1}$ prot; GR **(C)**: 1 unit = 1nmol NADPH oxidized $min^{-1}mg^{-1}$ prot. Data represent the means (±SE) of five experiments. Different letters represent values which are statistically different (by one-way Anova test).

total content of ASC increased significantly, reaching almost doubled values compared to T0 cells. T4 cells showed ASC levels and redox state fully comparable to those of T0 cells (de Pinto et al., 2006; Munné-Bosch et al., 2013).

The anoxic treatment caused a significant decrease of total glutathione content during the first 2 h of anoxia (T1)

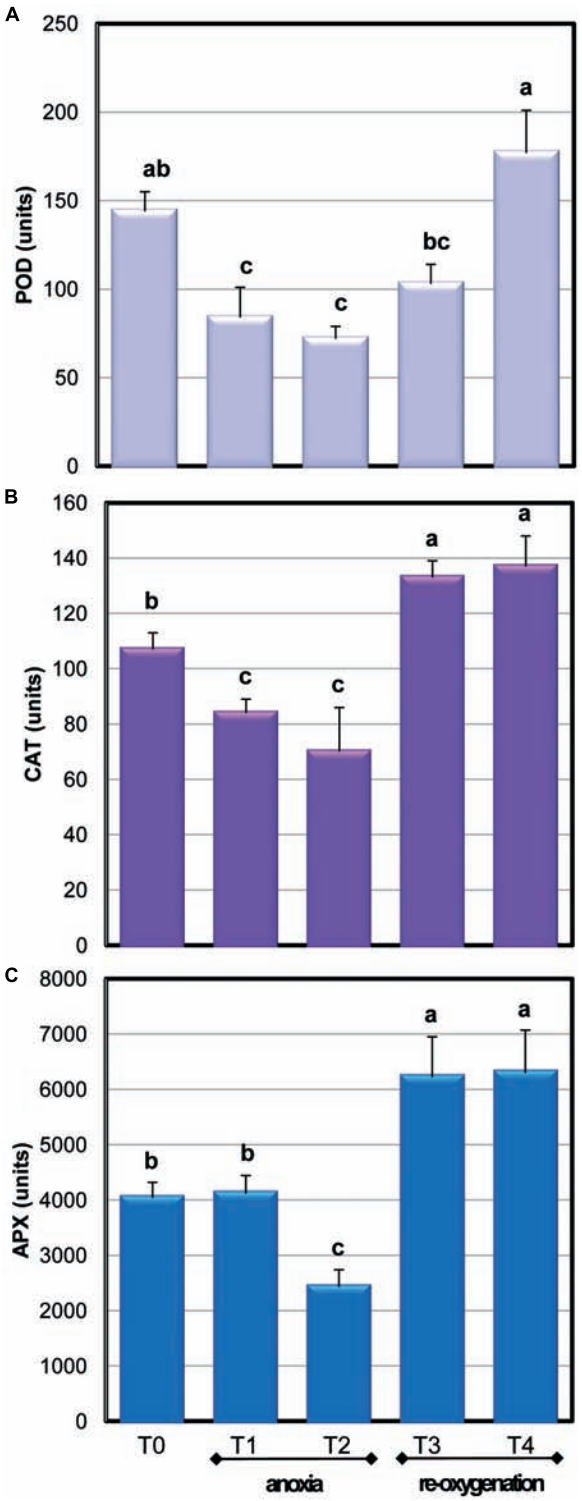

FIGURE 6 | Effects of anoxia and re-oxygenation on antioxidant enzymes in *A. thaliana* cell suspension cultures. Activities are expressed in units. **(A)** APX: 1 unit = 1nmol ASC oxidized $min^{-1}mg^{-1}$ prot; **(B)** CAT: 1 unit = 1nmol H_2O_2 dismutated $min^{-1}mg^{-1}$ prot; **(C)** POD: 1 unit = 1nmol TMB oxidated $min^{-1}mg^{-1}$ prot. Data represent the means (±SE) of five experiments. Different letters represent values which are statistically different (by one-way Anova test).

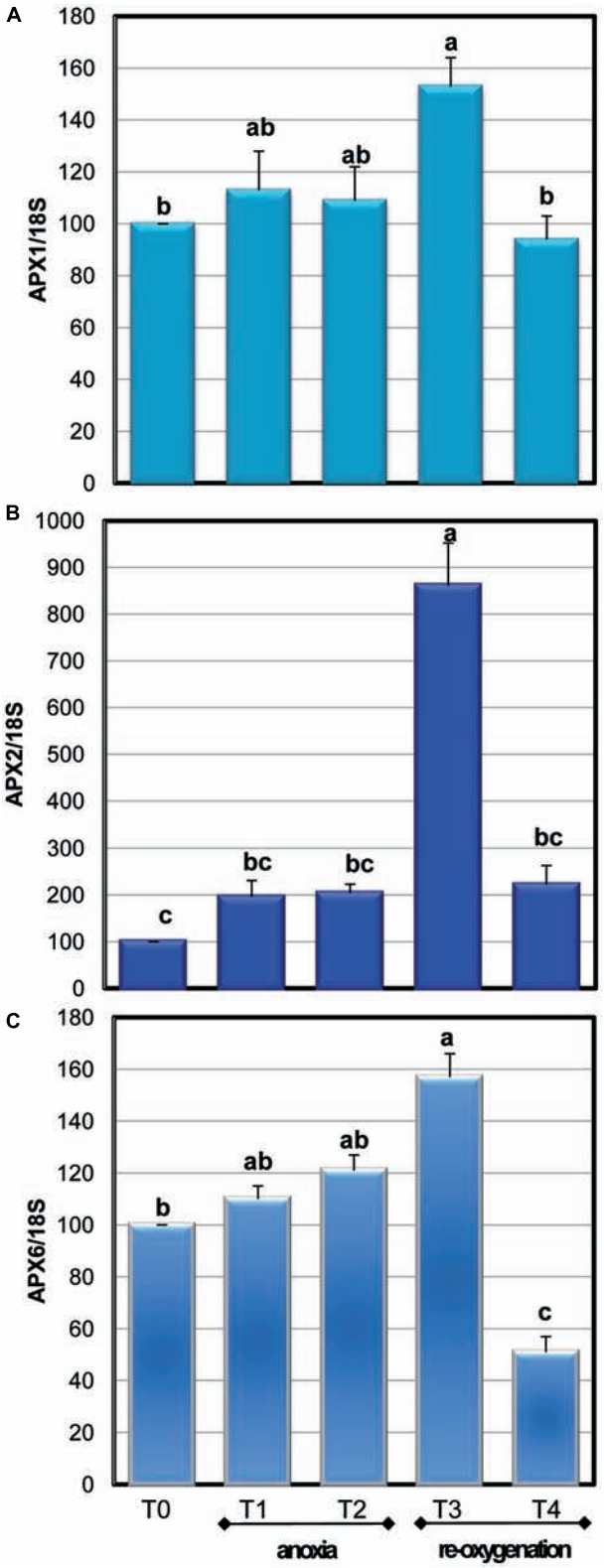

FIGURE 7 | Effects of anoxia and re-oxygenation on the expression of *APX1* (A), *APX2* **(B)**, and *APX6* **(C)** in *A. thaliana* cell suspension cultures. Gene expression changes were measured by RT-PCR using 18S rRNA as the internal control. Different letters represent values which are statistically different (by one-way Anova test).

(**Figure 4B**), while glutathione pool increased again to the T0 value in the following 2 h of anoxia. Differently from ASC, GSH redox state remained unchanged during oxygen concentration changes, with a ratio of more than 90% in reduced form. After re-oxygenation (T3), GSH content increased over threefold: the increase of ASC and glutathione pool, occurring at this stage, emphasized that the cells were responding to an extraordinary stress situation by enhancing their redox molecules. At T4 GSH returned to control values, similarly to what occurred for ASC, thus showing that cells were able to restore the physiological redox balance.

The levels of the lipophilic antioxidant metabolite α-tocopherol were also measured during normoxia, anoxia, and re-oxygenation (**Figure 4C**). In these experimental conditions, α-tocopherol showed a slight but significant increase at T2 phase and a slow decrease after re-oxygenation until reaching values similar to control cells (T4). In plants, α-tocopherol is known to increase in several environmental constraints involving oxidative stress (Munné-Bosch, 2005) thus an increase during anoxia could be expected. On the other hand, since ASC is involved in the process of regeneration of tocopherols, such regeneration during anoxia might likely contribute to the observed decrease in the ASC/DHA redox state.

Antioxidant Enzymes

To maintain the redox state and to regenerate antioxidant molecules in their active form, several enzymes act to support the antioxidative defense. The recycling enzymes of ASC-GSH cycle were tested in order to follow their behavior in *Arabidopsis* cell cultures at different oxygen concentrations (**Figure 5**). MDHAR and DHAR, the enzymes responsible for the reduction to ASC of MDHA and DHA, respectively, did not change significantly in response to both limited oxygen availability and following re-oxygenation. The activity of GR, enzyme responsible for glutathione reduction, significantly decreased during anoxia and gradually reached control values after re-oxygenation. It is worth noting that in spite of GR decreases during anoxia, the redox state of GSH pool remained high, thus suggesting that GR activity was overabundant.

To obtain information of the global H_2O_2 removal capability of *Arabidopsis* cells in response to oxygen concentration changes, the activity of APX, CAT, and POD was also measured (**Figure 6**). POD and CAT activity drastically decreased during anoxia to about 50% of the control value already after 2 h of oxygen deprivation; re-oxygenation induced a quick restoration of CAT activity while POD activity was completely restored only at the end of the experiment.

Among the APX isoenzymes, the activity of cytosolic ones (cAPX) was determined: under our experimental conditions, photosynthesis was not active (data not shown). Moreover, cytosol has been reported to act as key site of redox signaling integration (Noctor and Foyer, 2016). Anoxia resulted in a significant 40% decrease of cAPX activity only at T2. The high activity of this H_2O_2 scavenging enzyme during the first period of oxygen deprivation supports a key role for this enzyme in overcoming oxidative damage. During re-oxygenation, cAPX activity rapidly increased. This result is coherent with other evidences obtained when oxygen-deprived roots of wheat seedlings were re-aerated (Biemelt et al., 1998). Three genes encoding for cAPXs are present in *Arabidopsis* (Noctor et al., 2016). To test whether alteration in cAPX occurring during anoxia and re-oxygenation was due to gene expression changes, the transcript accumulation of these three genes was analyzed (**Figure 7**). The sequences of RT-PCR amplifications were compared to predicted mRNA sequences, confirming the correspondence with *Arabidopsis* genes (data not shown).

During anoxic treatment, the three c*APX* genes exhibited a similar pattern of transcript accumulation, showing no significant changes in their expression. Re-oxygenation induced an immediate increase in the transcript accumulation that was particularly high for APX2. This is consistent with the pivotal role of APX2 in responses to oxidative stress, since this isoenzyme is considered a stress marker and its expression is induced by several kinds of stresses (Gallas and Waters, 2015). Redox-regulated transcription factors have been characterized. The promoter of *Arabidopsis APX1* and *APX2* contains a heat shock element binding a heat shock factor sensitive to endogenous H_2O_2 production (de Pinto et al., 2015). In rice, an increase of H_2O_2 levels enhanced the expression of two cytosolic *APX* genes (Li et al., 2015). For all the three analyzed c*APX*s the expression values returned comparable to the control after 20 h of re-oxygenation. The increase in c*APX* gene expression observed when the anoxic cells were again exposed to oxygen might be related to the increase of enzyme activity occurring during re-oxygenation. On the other hand, no correlation was observed between the decrease of cAPX activity occurring at T2 and gene expression changes. It has been suggested that post-translational modifications are rapid regulators of enzyme activity. Most of these post-translational modifications are redox regulated and involve NO or ROS, such as carbonylation of specific amino acids, thiol – disulphide transition and *S*-nitrosylation (de Pinto et al., 2013; Waszczak et al., 2015). Due to the strong oxidative environment occurring during oxygen deprivation, it is likely that redox post-translational modifications are responsible for changes in enzyme activities, even if more in deep studies are required for verifying this hypothesis.

CONCLUSION

The results obtained in the present study using *Arabidopsis* suspension cell cultures grown in a stirred bioreactor indicate that imbalance of cell redox state occurs during the anoxic shock due to the dramatic increase of ROS, H_2O_2, and NO. On the other hand, the evaluation of cell defenses during anoxia and re-oxygenation and the absence of cell death suggest that the overproduction of reactive species triggers signaling pathways activating the antioxidant machinery such as ASC-GSH system, α-tocopherol and the antioxidant enzymes APX, CAT, and POD. The enhancement of these ROS-scavenging/controlling systems leads cells to counteract the stress toward cell survival, probably cooperating with other mechanisms controlling

ROS production and scavenging to cope with the metabolic impairment occurring during anoxia (Pucciariello and Perata, 2016). This study also indicates that bioreactors can be a useful tool for studying cell responses to qualitative and quantitative changes of chemical and physical parameters affecting cell life, thus opening new opportunities for monitoring the impact of environmental changes on cell metabolism.

AUTHOR CONTRIBUTIONS

AP performed biochemical and molecular analyses and contributed to drafting the manuscript. SC designed and coordinated the experimental work; wrote the manuscript. AL designed and conducted confocal microscopy analyses; contributed to drafting the manuscript. AB performed biochemical analyses. RN performed cell culture experimental work and HPLC analyses. LD analyzed and interpreted data; critically revised the manuscript for important intellectual content.

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Glutathionylation of Pea Chloroplast 2-Cys Prx and Mitochondrial Prx IIF Affects Their Structure and Peroxidase Activity and Sulfiredoxin Deglutathionylates Only the 2-Cys Prx

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Together with thioredoxins (Trxs), plant peroxiredoxins (Prxs), and sulfiredoxins (Srxs) are involved in antioxidant defense and redox signaling, while their regulation by post-translational modifications (PTMs) is increasingly regarded as a key component for the transduction of the bioactivity of reactive oxygen and nitrogen species. Among these PTMs, *S*-glutathionylation is considered a protective mechanism against overoxidation, it also modulates protein activity and allows signaling. This study explores the glutathionylation of recombinant chloroplastic 2-Cys Prx and mitochondrial Prx IIF from *Pisum sativum*. Glutathionylation of the decameric form of 2-Cys Prx produced a change in the elution volume after FPLC chromatography and converted it to its dimeric glutathionylated form, while Prx IIF in its reduced dimeric form was glutathionylated without changing its oligomeric state. Mass spectrometry demonstrated that oxidized glutathione (GSSG) can glutathionylate resolving cysteine (Cys^{174}), but not the peroxidatic equivalent (Cys^{52}), in 2-Cys Prx. In contrast, GSSG was able to glutathionylate both peroxidatic (Cys^{59}) and resolving (Cys^{84}) cysteine in Prx IIF. Glutathionylation was seen to be dependent on the GSH/GSSG ratio, although the exact effect on the 2-Cys Prx and Prx IIF proteins differed. However, the glutathionylation provoked a similar decrease in the peroxidase activity of both peroxiredoxins. Despite growing evidence of the importance of post-translational modifications, little is known about the enzymatic systems that specifically regulate the reversal of this modification. In the present work, sulfiredoxin from *P. sativum* was seen to be able to deglutathionylate pea 2-Cys Prx but not pea Prx IIF. Redox changes during plant development and the response to stress influence glutathionylation/deglutathionylation processes, which may represent an important event through the modulation of peroxiredoxin and sulfiredoxin proteins.

Keywords: 2-Cys peroxiredoxin, glutathione redox state, glutathionylation, peroxiredoxin IIF, post-translational modification, reactive nitrogen species, reactive oxygen species, sulfiredoxin

INTRODUCTION

The redox state of plant thiols and the regulation of cysteinyl residues in proteins are emerging as key players in the response of plants to both biotic and abiotic stresses (Sevilla et al., 2015a), functioning in redox sensing and signal transduction pathways. Reactive oxygen and nitrogen species (ROS/RNS) are known to act as signaling molecules in the maintenance of physiological functions and in the response to changing environments (Considine and Foyer, 2014). Plants are particularly exposed to oxidative and nitrosative stress, mainly due to their photosynthetic and respiratory metabolism, which can generate high levels of ROS/RNS under certain stress conditions (Martí et al., 2011; Lázaro et al., 2013). In the redox signaling process, protein thiols play a central role and redox-sensitive cysteines undergo a variety of post-translational modifications, including *S*-nitrosylation and glutathionylation, which are considered as an interesting point of control in the regulation of the protein structure and function (Hartl and Finkemeier, 2012; Zaffagnini et al., 2012a). Protein glutathionylation constitutes a reversible covalent post-translational modification (PTM) that takes place through the addition of glutathione to the thiolate of cysteines in target proteins. This modification is involved in many physiological processes, one of the most important being related to signaling, not only following oxidative or nitrosative stress but also under physiological, when thiolating agents are generated (Dalle-Donne et al., 2007). Another interesting aspect related to PTMs is the possible role as a mechanism for protecting proteins against modifications such as overoxidation (Roos and Messens, 2011). In more reducing conditions, deglutathionylation occurs as a result of the removal of the glutathione moiety from the protein, a process controlled by glutaredoxins (Grxs) and involving GSH and NADPH-dependent glutathione reductases (Meyer et al., 2012; Waszczak et al., 2015). Reversible protein glutathionylation is increasingly seen therefore not only as a major antioxidant defense against oxidative stress, but also as a cellular regulatory mechanism in cell signaling (Mieyal et al., 2008). In this context, ROS have been described as inducers of *S*-glutathionylation; more specifically, H_2O_2 plays an important role through its influence on the GSH/GSSG ratio, and is directly involved in the glutathionylation reaction, or through the direct oxidation of protein Cys, generating a thiyl intermediate which further reacts with GSH to form a mixed disulfide (Klatt and Lamas, 2000; Grek et al., 2013).

In cellular redox biology, there is growing interest in the involvement and regulation of the thioredoxin/peroxiredoxin/sulfiredoxin (Trx/Prx/Srx) system in plant signaling under abiotic stress conditions as an important cue that influences plant growth (Sevilla et al., 2015b). Among these redox proteins, Prxs are sensitive to glutathionylation. These ubiquitous thiol peroxidases have an antioxidant function, reducing H_2O_2, peroxynitrite and hydroperoxides. Mammals have six Prx isoforms (I-VI) grouped in three subfamilies, namely typical 2-Cys Prx (I-IV), atypical 2-Cys Prx (V) and 1-Cys Prx (VI), with different subcellular locations. In plants, they are localized in chloroplasts, mitochondria, nuclei, peroxisomes and cytosol, and are divided into four subgroups: 2-Cys Prx, type II Prx, Prx Q and 1-Cys Prx (Dietz, 2011). Typical chloroplast 2-Cys Prx and atypical mitochondrial Prx IIF have two characteristic cysteines involved in the reduction of peroxides, namely peroxidatic cysteine (Cp) and resolving cysteine (Cr). H_2O_2 oxidizes Cp to its sulfenic form that reacts with the Cr to form a disulfide bond that is reduced by thioredoxin (Trx), namely Trx*f* for chloroplast 2-Cys Prx and Trx*o* for mitochondrial Prx IIF (Barranco-Medina et al., 2008; Martí et al., 2009; Pulido et al., 2010). The difference in the reaction mechanism between both Prxs is the disulfide bond - intermolecular for chloroplast 2-Cys Prx and intramolecular for mitochondrial Prx IIF (Barranco-Medina et al., 2007; Dietz, 2011). There are also differences in their oligomeric states. Both Prxs form dimers, but while 2-Cys Prx forms decamers in its reduced or overoxidized state, Prx IIF only forms hexamers in its oxidized state (Barranco-Medina et al., 2009; Lázaro et al., 2013).

In severe oxidative stress conditions, Prxs are overoxidized to the inactive sulfinic form, which Srx, an ATP-dependent reductase located in chloroplasts and mitochondria, is able to retroreduce (Biteau et al., 2003). In fact, pea chloroplastic 2-Cys Prx and mitochondrial Prx IIF have been shown to be regenerated by pea Srx, which is then reduced by Trx (Iglesias-Baena et al., 2010, 2011). Scant information exists on the regulation of redox proteins by post-translational modifications, including the glutathionylation of chloroplastic 2-Cys Prx or mitochondrial peroxiredoxin IIF, aspects that will be addressed in this paper. 2-Cys Prx glutathionylation has been studied in the cytoplasmic protein of mammals but not in its chloroplastic counterpart of plants (Park et al., 2011; Chae et al., 2012), while human sulfiredoxin has been shown to catalyze the deglutathionylation of typical human 2-Cys Prx (Findlay et al., 2006; Park et al., 2009). In this work, the glutathionylation of both typical and atypical pea 2-Cys Prx and Prx IIF proteins is studied using reduced and oxidized glutathione (GSH, GSSG) and nitrosoglutathione (GSNO). The target cysteine residues and the oligomerization pattern after the treatments, as well as any effect on the peroxidase activity of both proteins, is analzed. In addition we study the capacity of pea Srx to deglutathionylate chloroplast 2-Cys Prx and mitochondrial Prx IIF.

MATERIALS AND METHODS

Cloning and Purification of Recombinant Proteins

Chloroplast 2-CysPrx and mitochondrial Prx IIF from pea (*P. sativum* L. cv. Lincoln grown as described in Barranco-Medina et al., 2007) were cloned without His-tag, and expressed and purified as described by Bernier-Villamor et al. (2004) and Barranco-Medina et al. (2006). The cloning, overexpression and purification of His-tagged sulfiredoxin (Srx) from pea was performed as described by Iglesias-Baena et al. (2010). Briefly, the fragment of cDNA encoding the mature proteins was obtained by reverse transcription-PCR and cloned into the pGEM-T (Promega, Madison, USA) (2-CysPrx) or pET3d (Novagen) (PsPrx IIF) or pETM-11 (PsSrx) expression vectors. *Escherichia coli* BL21 (DE3) strains were transformed with the resulting constructions, and recombinant protein expression was induced

by the addition of 1 mM isopropylthio-β-galactoside, leaving to stand for 3 h at 37°C. The *E. coli* cells were broken with a French press, followed by ammonium sulfate precipitation between 40 and 95% (w/v). The pellet was then suspended in buffer (25 mM Tris-HCl, pH 8.0, containing 150 mM NaCl) and chromatographed by FPLC.

Protein concentration was measured according to Bradford (1976) using bovine serum albumin as standard.

GSH, GSSG, GSNO, and SNP Treatment of Recombinant Proteins

One milliliter of 50 μM of purified recombinant protein (2-CysPrx or Prx IIF) was first reduced in 50 mM Tris-HCl pH 7.5 containing 10 mM DTT for 30 min at room temperature. The DTT excess was removed by Bio-Spin 6 gel filtration (BioRad).

For the analysis by gel filtration, 200 μL of reduced proteins preparations were incubated separately either with 5 mM GSSG at 4°C for 24 h or with 5 mM GSH, GSNO, or 750 μM SNP (sodium nitroprusside) prepared in 50 mM Tris-HCl pH 7.5 for 30 min at room temperature. After incubation, the excess of the modifying agent was removed by Bio-Spin 6 column and the samples were immediately analyzed through FPLC.

For the analysis of glutathionylation using different GSH/GSSG treatments, reduced proteins (15 μg) were incubated with different mM concentrations of GSH+GSSG as 4.987+0.0125, 4.95+0.05, 4.75+0.25, and 4.5+0.5 for 5 min at 37°C and the excess of glutathionylating agents was removed by Bio-Spin 6 gel filtration (BioRad). Also a 10 mM DTT-treated sample without any glutathionylating treatment was performed as negative control. Samples were immediately analyzed by western blot as described below, loading all the treated protein in each lane.

Gel Filtration Analysis

After treatment with GSSG, GSH, GSNO, and SNP, proteins were analyzed by gel filtration at room temperature using a Superdex-200 HR 10/30 column (GE Healthcare) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl at a flow of 0.5 mL/min. Absorbance at 280 nm was recorded and 250 μL fractions were collected. The peaks of each fraction (dimer and oligomer) were collected for mass spectrometry analysis. A calibration curve of the column was performed with albumin, chymotrypsinogen, ferritin, aldolase, ovalbumin, and ribonuclease as standards (Supplementary Figure S1).

Mass Spectrometry Analysis

Samples after FPLC chromatography were analyzed by mass spectrometry on an UltrafleXtreme Matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer (Bruker-Daltonics) in auto-mode using FlexControl v3.4 and processed using FlexAnalysis v3.4MALDI TOF/TOF apparatus (Bruker) as described in López-Vidal et al. (2016). Theoretical digestions were performed considering glutathionylation of cysteine in the peptide spectrum generated from the problem sample.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Denaturing SDS-PAGE was performed as described by Laemmli (1970) with acrylamide concentrations of 6% (staking gel) and 12.5% (resolving gel). Gels were stained with Coomassie Brilliant Blue R-250. For Western blot, proteins were transferred onto a nitrocellulose membrane by electroblotting. Pounceau S stained membranes were used as loading controls (Salinovich and Montelaro, 1986). Immunoreaction was performed with polyclonal antibodies against PsPrx IIF (1:3000) (Barranco-Medina et al., 2007), pea Srx peptide (CHRYEAHQKLGLPTI) (1:500) (Iglesias-Baena et al., 2010), pea 2-Cys Prx (1:5000) (Bernier-Villamor et al., 2004), and monoclonal anti-glutathione (1:500, Santa Cruz Biotechnology) diluted in TBS containing 1% (w/v) of BSA and 0.1% (v/v) of Tween-20. Anti-rabbit conjugated to alkaline phosphatase (1:7500, Boehringer Mannheim, Germany) and anti-mouse conjugated to peroxidase (1:5000, Santa Cruz Biotechnology) were used as secondary antibodies and the antigen was detected using the ECL-2 system (Thermo Scientific, USA), following the manufacturer's instructions.

Peroxidase Activity

Recombinant 2-Cys Prx and Prx IIF (100 μg) were treated with 10 mM DTT at room temperature for 30 min. The excess of DTT was then removed by Bio-Spin 6 gel filtration (BioRad, Spain) and 50 μg of protein were treated with 5 mM GSSG and with 4 mM GSH + 1 mM GSSG at 37°C for 5 min. The excess of GSSG and GSH was removed by Bio-Spin 6 gel filtration. Treated proteins (10 μg) were incubated with 50 μM H_2O_2 for 10 min at 37°C and the reaction was stopped adding 2% (p/v) trichloroacetic acid (TCA). A blank of the reaction was performed using sample buffer instead of peroxiredoxin proteins, incubated with DTT, followed by gel filtration and incubated with H_2O_2 and stopped with TCA. H_2O_2 was quantified using 100 μL of the reaction mixed with 500 μL of eFOX medium according Cheeseman (2006). H_2O_2 was determined based on the difference in absorption at 550 nm using a standard curve that covered the range of 0–200 μM.

Deglutathionylation of 2-Cys Prx and Prx IIF by Srx

Fifty microgram of recombinant 2-Cys Prx and Prx IIF were reduced with DTT as described above in a final volume of 50 μL and subjected to Bio-Spin 6 gel filtration. Forty microliter of these reduced proteins were incubated with 5 mM GSSG at 4°C for 24 h and dialyzed again to eliminate the excess of GSSG. A final concentration of 3 μM of GSSG-treated peroxiredoxin was incubated with a final concentration of 6 μM of DTT-treated Srx for different times and the reactions were stopped adding the non-reducing SDS-buffer. Finally, 3 μg of treated proteins were subjected to western blot analysis.

Statistics

The results are the mean of three replicates from each experiment which were repeated at least two times. The significance of any differences between the mean values was determined by one-way

analysis of variance. The Tukey's test was used to compare the means. All error bars represent standard error (SE) of the mean. The asterisk above the bars indicates significant difference ($P < 0.05$).

RESULTS

Effect of Glutathionylation on the Oligomeric State of 2-Cys Prx

After gel filtration on Superdex-200 HR 10/30, untreated 2-Cys Prx protein appeared exclusively as a dimer (**Figure 1A**), but when reduced with DTT, the protein mainly changed to its decameric form. Treating this reduced form with GSSG produced two modifications (**Figure 1A**): the decamer eluted earlier and it was also dissociated to a dimer. The oligomerization pattern was also analyzed by native PAGE and Coomasie staining (**Figure 1B**). The 2-Cys Prx protein pre-reduced with DTT was treated with GSSG and the protein without any reducing treatment was used as control. The treatment with 1 and 5 mM GSSG was carried out at 37°C for 5 min to check whether the changes in the oligomeric pattern also occurred in these conditions. The treatment of the DTT-treated 2-Cys Prx protein with GSNO also showed an advance in the elution volume of the decamer and its concentration fell slowly as the dimer concentration increased (**Figure 2**), the change being dependent on the incubation time.

Mass spectrometry analysis of the decameric and dimeric forms eluted from the gel filtration of the 2-Cys Prx protein treated with both GSSG and GSNO pointed to the glutathionylation of the resolving Cys^{174} but not of the peroxidatic Cys^{52} in both oligomeric forms (**Table 1** and Supplementary Figure S2). To ascertain whether a nitrosylated form of 2-Cys Prx was susceptible to glutathionylation with GSH, the reduced protein was first treated with an *S*-nitrosylating agent, sodium nitroprusside (SNP). It was observed that the reduced decamer delayed its elution volume, unlike the glutathionylated protein that forwarded the elution volume as shown in **Figure 3**. The subsequent treatment with GSH showed a similar result to that obtained by direct modification with GSSG.

Effect of Glutathionylation on the Oligomeric State of Prx IIF

The reduction of Prx IIF by DTT provoked a change in the hexameric form of the protein to a dimeric one. Glutathionylation of the dimeric form with both GSSG and GSNO, showed a slight shift (advance) in its elution volume (**Figure 4**). In this case, mass spectrometry analysis pointed to the glutathionylation of both resolving and peroxidatic Cys (59 and 84) (**Table 2** and Supplementary Figure S3).

Effect of Different GSH/GSSG Treatments on the Glutathionylation of 2-Cys Prx and Prx IIF

DTT-reduced recombinant proteins (15 μg) were treated with different GSH+GSSG mM concentrations (4.987+0.0125, 4.95+0.05, 4.75+0.25, and 4.5+0.5) and a 10 mM DTT sample without any glutathionylating treatment was used as negative control of the experiment. As shown in **Figure 5**, increasing concentration of GSSG increased glutathionylation of both the oligomeric and dimeric forms of 2-Cys Prx. However, the opposite was observed for the Prx IIF, the glutathionylation of which decreased as GSH diminished, although in general, a higher signal of glutathionylation was observed for this protein compared to that of the 2-Cys Prx. A representative gel is shown for each protein and numbers above the signals represent the mean of the densitometric analysis of the bands corresponding to four independent experiments. Pounceau staining was used to correct the loading.

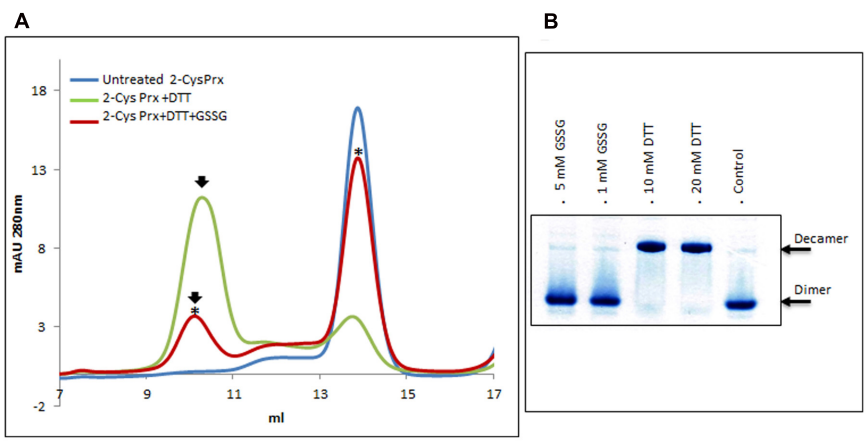

FIGURE 1 | Elution profile after size exclusion chromatography (A) through Superdex-200 HR 10/30 column of native (untreated) recombinant pea 2-Cys Prx and after its treatment with 10 mM DTT (+DTT) and 10 mM DTT + 5 mM GSSG (+DTT+GSSG). Asterisks indicate the samples subsequently analyzed by MALDI TOF/TOF. Pattern of oligomerization **(B)** analyzed by native PAGE and Coomasie staining of pre-reduced protein treated with GSSG or DTT, the non-treated protein (−DTT, −GSSG) being used as control.

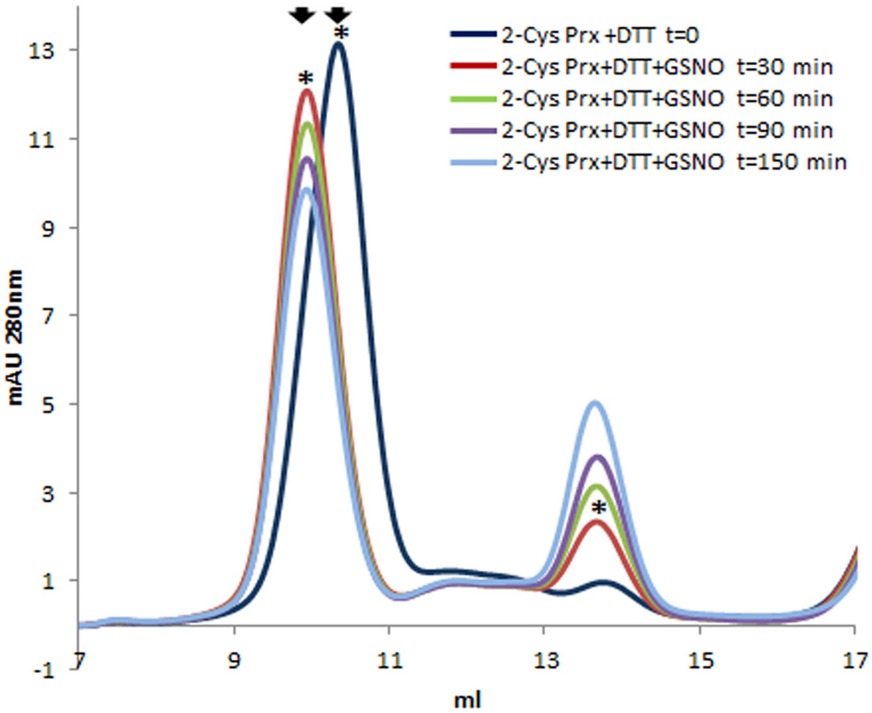

FIGURE 2 | Elution profile after size exclusion chromatography through Superdex-200 HR 10/30 column of pre-reduced pea 2-Cys Prx (+DTT) and after its treatment with 5 mM GSNO for different incubation times. Asterisks indicate the samples subsequently analyzed by MALDI TOF/TOF.

Effect of Glutathionylation on the Peroxidase Activity

Glutathionylation of 2-Cys Prx and Prx IIF led to a reduction in the peroxidase activity of the proteins, as represented by the H_2O_2 consumed in the reaction. This activity was measured in the DTT-reduced proteins (control) and after the treatment of the proteins with GSSG, as described above (**Figure 6**). The activity was found to be similarly reduced by the glutathionylation treatment, the reduction being approx 17% for both peroxiredoxin proteins. Similar results were found for a 4 mM GSH + 1 mM GSSG treatment (data not shown).

TABLE 1 | Glutathionylation of purified recombinant pea 2-Cys Prx analyzed by mass spectrometry MALDI-TOF/TOF after treatment of the pre-reduced (DTT) protein (2CPSH) with 5 mM GSSG or GSNO for 30 min (See Supplementary Figure S2).

Protein	Sample	Cysteine	Modification
2-Cys-Prx	2CPSH	C^{52}	NO
		C^{174}	NO
	2CPGSSG Decamer	C^{52}	NO
		C^{174}	**YES** 3105.40 (Cys-SG)
	2CPGSSG Dimer	C^{52}	NO
		C^{174}	**YES** 3105.43 (Cys-SG)
	2CPGSNO Decamer	C^{52}	NO
		C^{174}	**YES** 3105.46 (Cys-SG)
	2CPGSNO Dimer	C^{52}	NO
		C^{174}	**YES** 3105.39 (Cys-SG)

The incorporation of a SG group in the Cys^{52} or Cys^{174} in the decameric and dimeric forms of the protein eluted from the size exclusion chromatography through Superdex-200 HR 10/30 is pointed in bold.

Deglutathionylation of Glutathionylated Proteins by Srx

To assess whether the pea Srx protein was able to deglutathionylate both pea 2-Cys Prx and Prx IIF glutathionylated proteins, the proteins were first treated with GSSG and then both peroxiredoxins were incubated for different times with recombinant pea Srx protein previously reduced with DTT. After analysis of all the treated proteins by western blot using a specific GSH antibody, the deglutathionylation of 2-Cys Prx was found to be catalyzed by Srx, and the decrease in the glutathionylation was detected after 10 min (**Figure 7A**). However, Srx did not deglutathionylate Prx IIF in the analyzed conditions (**Figure 7B**). The loading was checked using a specific polyclonal 2-Cys Prx and Prx IIF antibodies.

DISCUSSION

Peroxiredoxins in non-photosynthetic organisms have been described as being among the proteins that are modified by the redox-sensitive mechanisms of glutathionylation and deglutathionylation, which, along with other post-translational mechanisms, are known to regulate their function, allowing

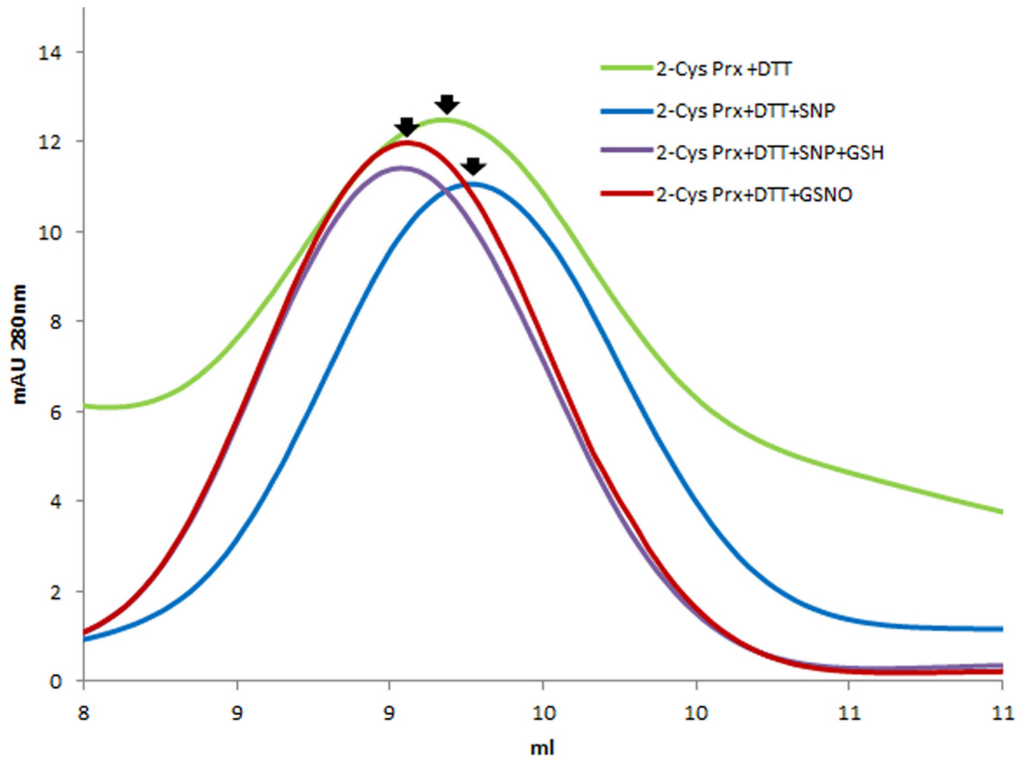

FIGURE 3 | Elution profile after size exclusion chromatography through Superdex-200 HR 10/30 column of DTT-reduced pea 2-Cys Prx and after its treatment with different *S*-nitrosylating and glutathionylating agents: 750 μM SNP, 750 μM SNP + 5 mM GSH and 5 mM GSNO.

localized H_2O_2 to build up, as described in the floodgate model (reviewed by Chae et al., 2012; Sevilla et al., 2015a). In plants, there is scarce information about glutathionylated proteins, although a 2 Cys-Prx has been described in *Arabidopsis* as being one of the proteins targets of this PTM (Dixon et al., 2005). Changes in the oligomeric pattern have been described as a consequence of PTMs of several proteins including peroxiredoxins from different origins, although the effect on chloroplastic 2-Cys Prx or mitochondrial Prx IIF is unknown. The transition from decamers to dimers of chloroplastic 2 Cys Prx due to glutathionylation, as observed in this study after treatment with GSSG and GSNO, is similar to that described for human cytosolic Prx I (Park et al., 2009, 2011). These authors reported that three of the four Prx I cysteine residues, Cys52, Cys83, and Cys173, were glutathionylated when treated with GSSG. Moreover, the glutathionylation of 50 μM Prx I was shown to promote changes in its quaternary structure from decamers (representing 97% of the total reduced protein) to mainly dimers with a higher peroxidase activity. This modification also provoked the inactivation of its molecular chaperone function mainly through the glutathionylation of a Cys^{83} located at the dimer–dimer interface and probably involved in the stabilization of the decamers (Chae et al., 2012). In this way, glutathionylation is able to alter the structure and thus the function of this antioxidant protein (Park et al., 2011), with possible implications in situations involving redox changes or oxidative/nitrosative stress. On the other hand, human Prx II, another cytosolic 2-Cys Prx which lacks Cys^{83}, has been described as being less susceptible to glutathionylation by glutathione than Prx I, which may not be easily accessible to interact with the peroxidatic and resolving Cys^{52} and Cys^{173} in the dimer (Park et al., 2011). The chloroplast 2-Cys Prx studied in the present work has two cysteines, Cys^{52} and Cys^{174}, the latter resolving Cys being glutathionylated. The observed change in the elution profile of the decamer after FPLC Superdex-200 HR 10/30 chromatography of the glutathionylated protein could be due to a conformational change, because the possible change in molecular mass as a result of the addition of GSH would not seem to justify this behavior. On the other hand, glutathionylation of this Cys seems to destabilize the decamer and the protein is present mainly as a dimer. Any structural change in Prxs may affect their redox state, oligomeric structure, and/or interaction with other proteins and could have a significant impact on the cascade of H_2O_2-related signaling events (Sevilla et al., 2015a). It has been described that functional 2-Cys Prx is a dimer (Dietz, 2011) and we have found that glutathionylation of both the decamer and dimer forms negatively affected peroxidase activity strongly suggesting that glutathionylation affects the function of this protein in chloroplasts. This is especially interesting taking into account the recent suggestion concerning the chaperone function of plant 2-Cys Prx, which does not seem to be essential in planta, because of the absence of high-molecular weight complexes under severe but

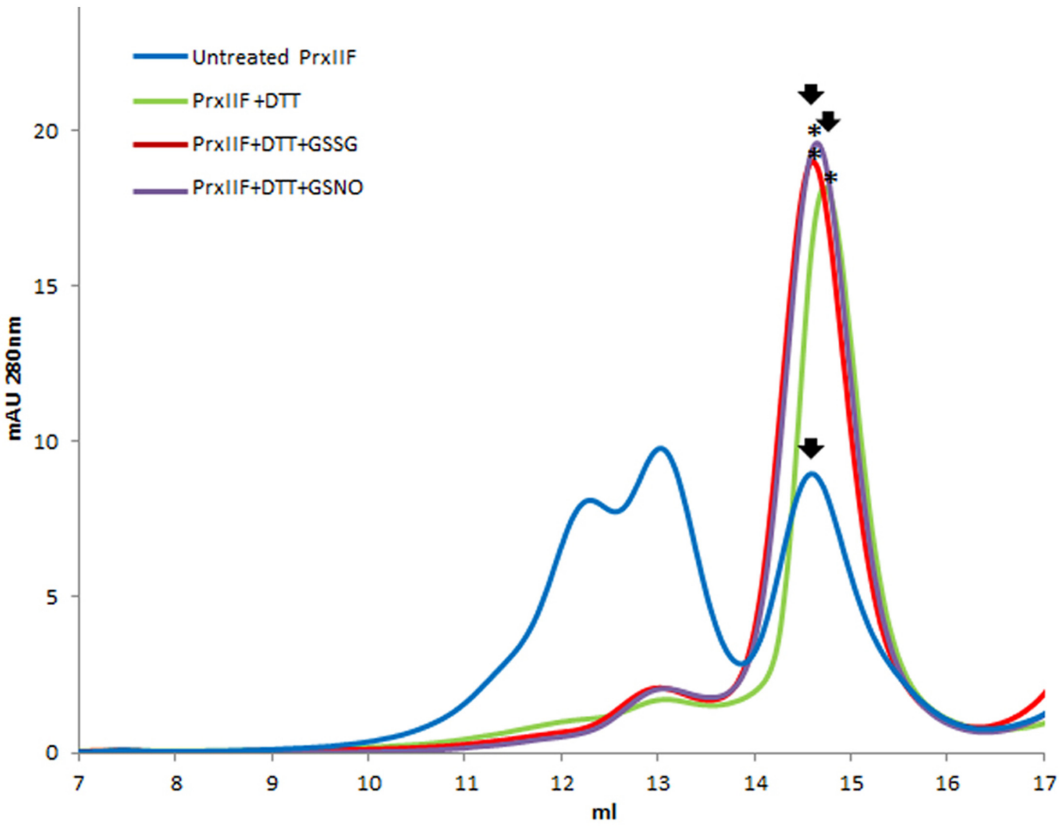

FIGURE 4 | Elution profile after size exclusion chromatography through Superdex-200 HR 10/30 column of native (untreated form) recombinant pea Prx IIF and after its treatment with 10 mM DTT and 5 mM GSSG and GSNO. Asterisks indicate the samples subsequently analyzed by MALDI TOF/TOF.

physiological water deficit and photooxidative stress conditions, highlighting the peroxidase activity of this protein (Cerveau et al., 2016).

As regard PsPrx IIF, glutathionylation did not induce a change in its oligomeric state but produced a similar shift in the elution profile to that recently described for *S*-nitrosylation of the protein after GSNO and SNP treatments: an advance in the elution volume of both the hexameric and dimeric forms (Camejo et al., 2015). While the effect of *S*-nitrosylation was described as decreasing the peroxidase activity of Prx IIF, which acquired a new transnitrosylase activity on its target protein, citrate synthase, the effect of glutathionylation of this protein has not been evaluated before. The decreased peroxidase activity found following the glutathionylation of both Cys residues in Prx IIF by GSNO and GSSG points to an additional post-translational modification of this peroxidase in the mitochondria that would influence its role in redox control, with potential implications for cell signaling.

TABLE 2 | Glutathionylation of purified recombinant pea Prx IIF analyzed by mass spectrometry MALDI-TOF/TOF after treating the pre-reduced (DTT) protein (IIFSH) with 5 mM GSSG or GSNO for 30 min.

Protein	Sample	Cysteine	Modification
Prx IIF	IIFSH	C^{59}	NO
		C^{84}	NO
	IIFGSSG	**C^{59}**	**YES** 2002.94 (Cys-SG) 2131.06 (Cys-SG)
		C^{84}	**YES** 2669.20 (Cys-SG) 2868.33 (Cys-SG)
	IIFGSNO	**C^{59}**	**YES** 2002.96 (Cys-SG) 2131.04 (Cys-SG)
		C^{84}	**YES** 2669.20 (Cys-SG) 2868.33 (Cys-SG)

The incorporation of a SG group in the Cys^{59} or Cys^{84} in the dimeric form of the protein eluted from the size exclusion chromatography through Superdex-200 HR 10/30 is pointed in bold.

Protein glutathionylation is primarily influenced by the glutathione redox state and the most studied mechanism of glutathionylation is the spontaneous thiol/disulfide exchange between GSSG and a protein cysteine thiol (Zaffagnini et al., 2012b). Reduced glutathione concentration in organelles such as chloroplasts and mitochondria are described to be around 1-5 mM and 6-10 mM, respectively (Foyer and Halliwell, 1976; Law et al., 1983; Bielawski and Joy, 1986; Noctor et al., 1998; Koffler et al., 2013). The glutathione pool is kept highly reduced by glutathione reductase and the relationship between GSH and GSSG are usually in the range of 95% GSH 5% GSSG, although Zaffagnini et al. (2012b) described a GSH/GSSG ratio of around 10^5. Moreover, these authors described that a change in the GSH/GSSG to 1 (K_{ox} of the Cys: a value that is thought to be the range at which 50% of the proteins could be glutathionylated), was unlikely to have occurred during

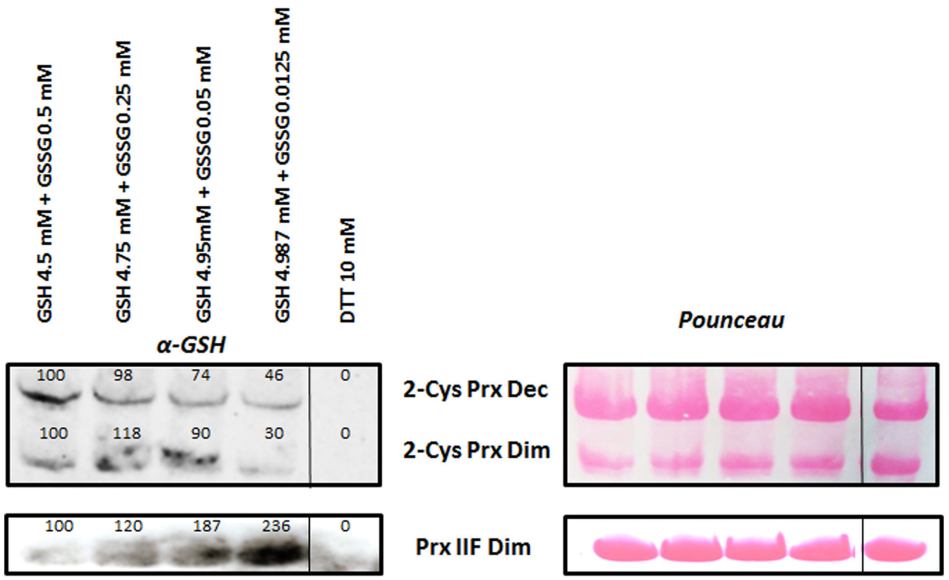

FIGURE 5 | Glutathionylation of recombinant 2-Cys Prx and Prx IIF by different concentrations of GSH/GSSG. DTT-reduced Prx proteins were incubated with the different concentrations for 5 min at 37°C and the excess of glutathionylating agents was removed. A DTT-treated sample was used as negative control. Samples (15 μg protein) were immediately analyzed by western-blot using a specific monoclonal glutathione antibody, and a representative example is shown. Numbers show the mean of the densitometric analysis of at least 4 independent experiments, relative to the first band in each of the forms of the proteins. Pounceau S stained membranes were used as loading controls. Dec: decameric and Dim: dimeric forms of the proteins.

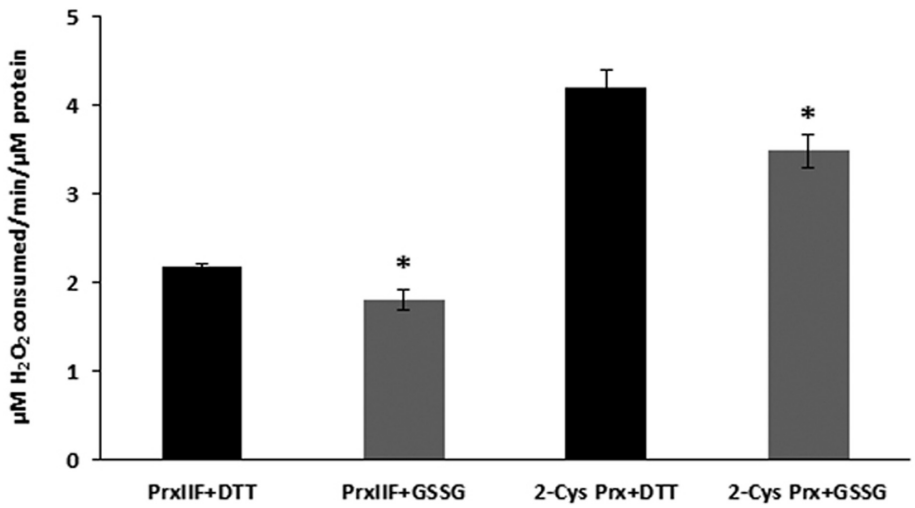

FIGURE 6 | Peroxidase activities of recombinant 2-Cys Prx and Prx IIF after the treatment with 5 mM GSSG. Peroxidase activity was measured in previously DTT-reduced proteins (control) and in GSSG-treated proteins after incubation with H_2O_2 for 10 min at 37°C, using trichloroacetic acid to stop the reactions, as described in material and methods. H_2O_2 was then quantified using the eFOX method.

stress. However, a chloroplastic GRX of poplar has a K_{ox} of 309 and might be glutathionylated *in vivo* under physiological stress conditions (Couturier et al., 2009). On the other hand, it has been reported that the GSH/GSSG ratio influences the extent of oxidation of mitochondrial protein thiols by GSSG (Beer et al., 2004). Thus, in order to determine whether the glutathionylation of 2-Cys Prx and Prx IIF was dependent on this ratio, the assays were performed in the presence of different GSH/GSSG ratios, and the results were different for the two proteins, with an increase in the glutathionylation of the 2-Cys Prx and Prx IIF dependent on increasing concentrations of GSSG and GSH, respectively, with a higher amount of PrxIIF protein being glutathionylated in the assayed conditions. This different behavior is interesting taking into account the different subcellular location of both peroxiredoxins and the different susceptibilities of the Cys to be glutathionylated, as well as

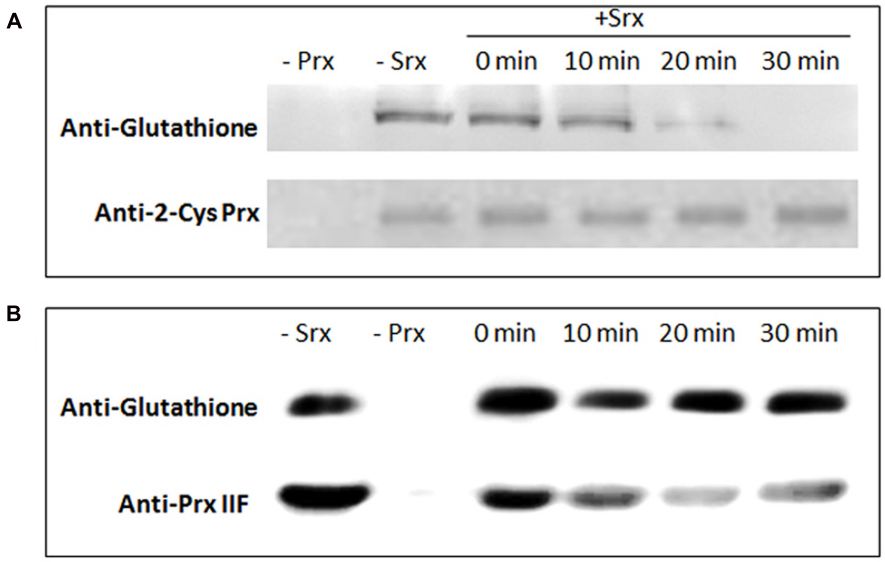

FIGURE 7 | Deglutathionylation of recombinant 2-Cys Prx (A) and Prx IIF **(B)** proteins by pea recombinant Srx. Prx proteins were treated with 5 mM GSSG and then incubated for different times with recombinant DTT-treated Srx. The samples (3 μg of protein) were analyzed by western-blot using a monoclonal glutathione antibody. The loading was checked using specific polyclonal 2-Cys Prx and Prx IIF antibodies.

the different effect on the oligomerization of the proteins. *S*-glutathionylation seems to be dependent on the different sensitivity of the Cys residues to the glutathionylating agent although further studies will be necessary to determine the exact mechanism underlying this different effect. Anyway, it is important to point out that very small changes in GSH/GSSG during cellular metabolism but mainly during stress could play a key role in signaling events, while the fact that 2-Cys Prx and Prx IIF thiol glutathionylation was sensitive to these changes points to a fine regulation by a mild oxidation of the glutathione pool. In light of the rapid response to this PTM, both peroxiredoxins may contribute to the antioxidant defense in chloroplasts and mitochondria, regulating H_2O_2 concentration and signaling.

The interplay between nitrosative and oxidative stress could be through PTMs which may lead to a conformational change in the proteins that could prevent their overoxidation or carbonylation and thus the irreversible loss of function. This point of control might be especially important for an adequate plant response (Tanou et al., 2009; Camejo et al., 2013). More specifically an interplay between *S*-nitrosylation and *S*-glutathionylation exists, the physiological agent GSNO being able to produce both modifications. As an example, human eNOS has been described as being regulated by glutathionylation (Chen et al., 2010). To check whether a nitrosylated form of 2-Cys Prx could also be glutathionylated, the protein was first treated with an *S*-nitrosylating agent (SNP) before glutathionylation with GSH. The result was similar to that obtained with the direct treatment with GSNO, suggesting that glutathionylation of the decamer could be caused directly by GSSG or GSNO or indirectly by GSH on a nitrosylated form of the protein (**Figure 8**); in fact, GSH has been seen to glutathionylate –SOH or -SNO groups (Zaffagnini et al., 2012b). Once glutathionylated, the decamer would change

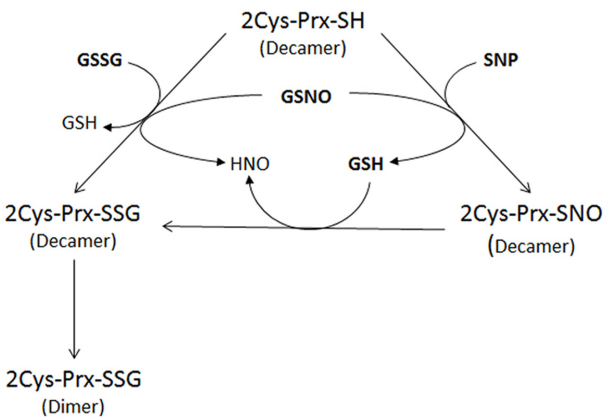

FIGURE 8 | Proposed mechanism of glutathionylation of the decameric reduced form of 2-Cys Prx after treatment with 5 mM GSSG, 5 mM GSNO or 750 μM SNP. Glutathionylation of the 2-Cys Prx can be caused directly by GSSG or GSNO or indirectly by GSH after nitrosylation of the protein by SNP. The glutathionylation of the decameric 2-Cys Prx induces dimerization of the protein.

to the dimeric form, probably as a result of the conformational instability of the decameric glutathionylated form. It has been described that NO-induced GSH oxidation may contribute to RNS-induced protein thiolation. The reaction of GSH with protein thiols that are *S*-nitrosylated upon exposure to RNS converts nitrosylated cysteines into relatively more stable mixed disulfides (Klatt and Lamas, 2000). On the other hand, many of the proteins reported as *S*-thiolated under oxidative conditions have subsequently been described as being susceptible to the formation of a mixed disulfide in response to RNS. Therefore

the *S*-glutathionylation of active-site cysteines may integrate oxidative and nitrosative stress via redox-dependent and/or redox-independent (through GSNO) mechanisms (Klatt and Lamas, 2000).

Different results have been attributed to the capacity of Srx to deglutathionylate many protein targets *in vitro* and *in vivo* following oxidative and/or nitrosative stress (Findlay et al., 2006), including Prxs, depending on the glutathionylating agent. In the present work, plant sulfiredoxin deglutathionylated GSSG-treated 2-Cys Prx and not Prx IIF. Human Srx has also been described to deglutathionylate GSSG-treated Prx I, a ubiquitous 2-Cys Prx, but not Prx V, an atypical 2-Cys Prx (like plant Prx IIF). It has been described that Cys^{83} and Cys^{173} residues were preferentially deglutathionylated by Srx, and glutathionylated Srx was found as intermediate, which was rapidly deglutathionylated by GSH, whereas glutaredoxin I deglutathionylated Cys^{52} (Park et al., 2009). The fact that pea chloroplastic/mitochondrial Srx is not able to deglutathionylate Prx IIF implies that glutaredoxin might be a potential key protein in the mitochondria and thus in ROS/RNS functionality in this organelle, an aspect that merits further attention, while in chloroplast, the Srx protein could play a central role in the redox control. Both proteins would be involved in cell signaling in oxidative or nitrosative environments as a result of their glutathionylation/deglutathionylation, which may influence protein function, affecting among others, the H_2O_2 or hydroperoxide levels.

CONCLUSION

The glutathionylation of pea chloroplastic 2-Cys Prx and mitochondrial Prx IIF induced a change in their structure but also in the oligomerization state of the chloroplastic enzyme. The peroxidase activity of both proteins was similarly reduced by glutathionylation, which was detected in the resolving cysteine of 2-Cys Prx and in both Cys of the Prx IIF protein. Glutathionylation was dependent on the GSH/GSSG ratio, which affected both proteins differently, and sulfiredoxin was able to deglutathionylate 2-Cys Prx but not Prx IIF. In this way, glutathionylation may act, on the one hand, as a temporary protection of peroxiredoxins in physiological processes in which oxidative and/or nitrosative stress are involved and, on the other hand, this PTM could play a significant role in situations where H_2O_2 acts as a signaling molecule, modulating the peroxidase activity of these proteins. Studies of the biological relevance of the glutathionylation-deglutathionylation processes *in vivo* for peroxiredoxin proteins and their involvement during plant development or stress response are being conducted in order to establish the significance of these modifications.

AUTHOR CONTRIBUTIONS

JL, FS, and AJ designed research. AC, AL-P, II-B, and DC carried out research. JL, FS, and AJ wrote and revised the manuscript. All authors discussed the results and commented the manuscript and have given approval to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00118/full#supplementary-material

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Hydrogen Peroxide Pretreatment Mitigates Cadmium-Induced Oxidative Stress in *Brassica napus* L.: An Intrinsic Study on Antioxidant Defense and Glyoxalase Systems

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Cadmium (Cd) is considered as one of the most toxic metals for plant growth and development. In the present study, we investigated the role of externally applied hydrogen peroxide (H_2O_2) in regulating the antioxidant defense and glyoxalase systems in conferring Cd-induced oxidative stress tolerance in rapeseed (*Brassica napus* L.). Seedlings were pretreated with 50 μM H_2O_2 for 24 h. These pretreated seedlings as well as non-pretreated seedlings were grown for another 48 h at two concentrations of $CdCl_2$ (0.5 and 1.0 mM). Both the levels of Cd increased MDA and H_2O_2 levels and lipoxygenase activity while ascorbate (AsA) declined significantly. However, reduced glutathione (GSH) content showed an increase at 0.5 mM $CdCl_2$, but glutathione disulfide (GSSG) increased at any level of Cd with a decrease in GSH/GSSG ratio. The activities of ascorbate peroxidase (APX) and glutathione *S*-transferase (GST) upregulated due to Cd treatment in dose-dependent manners, while glutathione reductase (GR) and glutathione peroxidase (GPX) increased only at 0.5 mM $CdCl_2$ and decreased at higher dose. The activity of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), catalase (CAT), glyoxalase I (Gly I), and glyoxalase II (Gly II) decreased under Cd stress. On the other hand, H_2O_2 pretreated seedlings, when exposed to Cd, AsA and GSH contents and GSH/GSSG ratio increased noticeably. H_2O_2 pretreatment increased the activities of APX, MDHAR, DHAR, GR, GST, GPX, and CAT of Cd affected seedlings. Thus enhancement of both the non-enzymatic and enzymatic antioxidants helped to decrease the oxidative damage as indicated by decreased levels of H_2O_2 and MDA. The seedlings which were pretreated with H_2O_2 also showed enhanced glyoxalase system. The activities of Gly I, and Gly II and the content of GSH increased significantly due to H_2O_2 pretreatment in Cd affected seedlings, compared to the Cd-stressed plants without H_2O_2 pretreatment which were

vital for methylglyoxal detoxification. So, the major roles of H_2O_2 were improvement of antioxidant defense system and glyoxalase system which protected plants from the damage effects of ROS and MG. The mechanism of H_2O_2 to induce antioxidant defense and glyoxalase system and improving physiology under stress condition is not known clearly which should be elucidated. The signaling roles of H_2O_2 and its interaction with other signaling molecules, phytohormones or other biomolecules and their roles in stress protection should be explored.

Keywords: abiotic stress, antioxidant defense, cross tolerance, metal toxicity, methylglyoxal, oxidative stress, signaling molecule

INTRODUCTION

Metal toxicity has been increasing considerably due to increase of toxic metal release as a result of urbanization and industrialization (Hasanuzzaman and Fujita, 2012). Cadmium (Cd) is considered as most toxic considering injurious effects on plant developmental processes and metabolism (Nouairi et al., 2009). Cd has nature to enter through roots readily and easily due to its high solubility in water. Cd content higher than 5–10 μg Cd g^{-1} leaf dry weight is considered toxic for plants, in general (White and Brown, 2010). Cd stress reduces growth and metabolism affecting plants' basic physiological processes including water and nutrient translocation and assimilation, transpiration and photosynthesis (Hasanuzzaman and Fujita, 2012; Khan et al., 2015). At cellular level Cd provokes generation of ROS [may include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and so forth] which results in oxidative damages to lipids, proteins and fatty acid which disrupt biomembrane, ultrastructural cellular components, DNA, and causes programmed cell death (PCD; Gill and Tuteja, 2010; Nahar et al., 2015, 2016a). Plants' antioxidant system scavenges ROS and keeps a state of balance under non-stress condition. Antioxidant machinery posses non-enzymatic antioxidants [AsA, GSH, flavonoids, phenolic compounds, carotenoids, alkaloids, Pro, non-protein amino acids, and α-tocopherols] and a bunch of antioxidant enzymes [CAT, APX, MDHAR, DHAR, GR, GPX, guaiacol peroxidase, and GST] which works in coordinated manner to scavenge ROS and to minimize oxidative stress (Apel and Hirt, 2004; Hasanuzzaman et al., 2012b). MG generation is an impulsive outcome of the glycolysis. Due to environmental stresses MG is overproduced many times higher than the normal growth condition to create toxic effects (Kaur et al., 2015). In glyoxalase system, utilizing GSH MG is transformed in to SLG by the activity of Gly I, while Gly II transforms SLG to D-lactic acid which is a MG detoxification process. At the end, GSH is regenerated. Tolerance against ROS and MG confers and improves abiotic stress adaptation and tolerance in different plants (Yadav et al., 2008; Hasanuzzaman et al., 2012a; Kaur et al., 2015).

Among the ROS, H_2O_2 has stability, being a versatile molecule shows signaling function (Quan et al., 2008; Saxena et al., 2016). It takes part of oxidative metabolism. It has been proved to involve in signaling cascades and metabolism which are vital for plants growth/developmental processes. Seed germination, initiation of root hair, strengthening of cell wall, cell wall loosening, xylem differentiation and stomatal movement were reported to link with H_2O_2 mediated signaling cascade (Dempsey and Klessig, 1995; Wojtyla et al., 2016). Interacting with other hormones and signaling molecules [abscisic acid (ABA) and ethylene], H_2O_2 regulates plant metabolism (Jubany-Mari et al., 2009; Chen et al., 2012). Recently it has been reported that nitric oxide (NO) and H_2O_2 regulate the salicylic acid (SA)-induced salvianolic acid B production (Guo et al., 2014). Thus, as a signaling molecule H_2O_2 regulates different metabolic pathways to develop stress tolerances (Mittler et al., 2004; Reczek and Chandel, 2015). H_2O_2-induced signal stimulates the expression and activation of stress tolerant genes (Prasad et al., 1994) which mediate stress acclimation and adaptation (Uchida et al., 2002). In different research findings, H_2O_2 mediated chilling (Prasad et al., 1994), salinity (Xu et al., 2008; Li et al., 2011), heat (Gao et al., 2010), osmotic stress (Liu et al., 2010), Cd (Hu et al., 2009), low light (Zhang et al., 2011), and multiple stress (Gong et al., 2001) tolerances were reported. Based on the results of previous studies we hypothesize that application of exogenous H_2O_2 might have a signaling function, influence antioxidant activities which can improve Cd stress tolerance. Very few research works demonstrated the beneficial roles of H_2O_2 on Cd or heavy metal stress (Chao et al., 2009; Hu et al., 2009). In the previous study, only few components of antioxidant defense system have been examined to show the effect of H_2O_2 under Cd stress (Chao et al., 2009; Hu et al., 2009). Moreover, effects of H_2O_2 on MG detoxification system under Cd stress were not reported. Many aspects of H_2O_2-induced Cd stress tolerance are yet to be elucidated. The present study provides a new insight into H_2O_2-induced coordinated effects on antioxidant defense and glyoxalase system to enhance the resistance to

Abbreviations: AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, ascorbic acid (ascorbate); BSA, bovine serum albumin; CAT, catalase; CDNB, 1-chloro-2, 4-dinitrobenzene; Chl, chlorophyll; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Gly I, glyoxalase I; Gly II, glyoxalase II; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; HSD, honest significant difference; LOX, lipoxygenase; MDA, malondialdehyde; MDHA, monodehydroascorbate; MDHAR, MDHA reductase; MG, methylglyoxal; NADPH, nicotinamide adenine dinucleotide phosphate; NTB, 2-nitro-5-thiobenzoic acid; PEG, polyethylene glycol; Pro, proline; ROS, reactive oxygen species; RWC, relative water content; SLG, *S*-D-lactoylglutathione; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

Cd toxicity in rapeseed seedlings. In this study, we will present several components of antioxidant defense and MG detoxification systems which were not mentioned in previous research findings.

MATERIALS AND METHODS

Plant Material, Growth Condition, and Treatments

Healthy and uniform sized rapeseed (*Brassica napus* cv. BINA sharisha 3) seeds were dipped into 70% ethanol for 5 min, then washed with double distilled water (ddH_2O). Seeds had been sown in Petri plates (9 cm) containing six layers of filter paper where filter papers were provided with 10 ml of ddH_2O. The Petridishes containing seeds were kept in a dark germination chamber under controlled conditions, 72 h. Germinated seedlings were removed from the germinator and placed into growth chamber under control environment (providing with light 100 μmol photon m^{-2} s^{-1}, temp 25 ± 2°C, RH 65–70%). Seedlings were supplied with 10,000-fold diluted Hyponex solution (Hyponex, Japan) as nutrient at regular interval. Eleven-day-old seedlings were pretreated with 50 μM H_2O_2 in their root for 24 h. Both H_2O_2-pretreated and non-pretreated seedlings were then exposed to Cd stress (0.5 and 1.0 mM $CdCl_2$) for 48 h. Several trial experiments were conducted before selecting the present doses of treatments. Different doses of Cd were applied in combination with different doses of H_2O_2 and the present combination (0.5 and 1.0 mM $CdCl_2$ with 50 μM H_2O_2; 48 h) showed the better result. We hypothesized that using two concentrations of Cd the trend how the H_2O_2 is affecting the Cd-stressed rapeseed seedlings could be understood better. The same experiment was repeated three times under the same treatment condition. There were 45 seedlings in each Petri dish. In total $6 \times 3 = 18$ dishes were used.

Measurement of Lipid Peroxidation

Lipid peroxidation had been determined by estimating MDA (a product of lipid peroxidation) using TBA (Heath and Packer, 1968; Hasanuzzaman et al., 2011).

Measurement of Hydrogen Peroxide Content

Hydrogen peroxide (H_2O_2) had been determined extracting leaves in potassium phosphate (K-P) buffer (pH 6.5; centrifuging at 11,500×*g*), then adding it to a mixture of $TiCl_4$ in 20% H_2SO_4 (v/v). The supernatant was read spectrophotometrically at 410 nm (Yu et al., 2003).

Histochemical Detection of Hydrogen Peroxide and Superoxide

The H_2O_2 and $O_2^{\bullet-}$ were determined histochemically (Chen et al., 2010) in the leaves of rapeseed plants by staining leaves with 1% 3,3-diaminobenzidine (DAB; to get brown spots due to the reaction of DAB with H_2O_2) and 0.1% nitroblue tetrazolium chloride (NBT; to get deep blue spots appeared due to the reaction of NBT with $O_2^{\bullet-}$) solution, respectively. Then, leaves were blanched in boiling ethanol to visualize the spots.

Extraction and Measurement of Ascorbate and Glutathione

The leaves of rapeseed plant (0.5 g) had been homogenized in 5% meta-phosphoric acid containing 1 mM EDTA (centrifuged at 11,500 × *g*; 15 min at 4°C). Supernatant was collected for the assay of AsA and GSH pool. To determine total ascorbate, the oxidized fraction was reduced by adding 0.1 M dithiothreitol for 1 h at room temperature and then read at 265 nm using 1.0 unit AO. Oxidized ascorbate (DHA) content had been assayed by subtracting reduced AsA from total AsA (Hasanuzzaman et al., 2011; Nahar et al., 2016b). The glutathione pool had been determined according to previously described methods (Yu et al., 2003; Hasanuzzaman et al., 2011). Standard curves with known concentrations of GSH and GSSG had been used to calculate the unknown GSH and GSSG pool of plant sample. The content of reduced GSH had been calculated by subtracting GSSG from total GSH.

Protein Determination

Following the method of Bradford (1976) the protein content had been measured where we used BSA as a protein standard.

Enzyme Extraction and Assays

Leaves had been homogenized with 50 mM K-P buffer (pH 7.0) containing 100 mM KCl, 1 mM AsA, 5 mM β-mercaptoethanol, and 10% (w/v) glycerol in pre-chilled mortars. Homogenates were centrifuged at 11,500 × *g*. The supernatants were collected and used for the assay of enzyme activity.

Ascorbate peroxidase (EC: 1.11.1.11) activity: The reaction buffer solution contained 50 mM K-P buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract (final volume 700 μL). The reaction had been initiated adding H_2O_2. Absorbance had been monitored at 290 nm for 1 min and activity has been calculated using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ (Nakano and Asada, 1981).

Monodehydroascorbate reductase (EC: 1.6.5.4) activity: The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 unit of AO, and enzyme solution (final volume 700 μL). The reaction had been started by adding AO. Absorbance was taken at 340 nm; activity had been calculated from the change in absorbance for 1 min using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ (Hossain et al., 1984).

Dehydroascorbate reductase (EC: 1.8.5.1) activity: The reaction buffer contained 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. Activity had been calculated from the change in absorbance at 265 nm for 1 min using an extinction coefficient of 14 $mM^{-1}cm^{-1}$ (Nakano and Asada, 1981).

Glutathione reductase (EC: 1.6.4.2) activity: The reaction mixture contained 0.1 M K-P buffer (pH 7.0), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and enzyme solution (final volume 1 mL). The reaction had been started with GSSG; the decrease in absorbance at 340 nm was monitored for 1 min and activity had

been calculated using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ (Hasanuzzaman et al., 2011).

Glutathione *S*-transferase (EC: 2.5.1.18) activity: The reaction mixture had 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM CDNB, and enzyme solution (final volume 700 μL). The reaction had been started by CDNB; the raise of absorbance was monitored at 340 nm for 1 min. Activity had been calculated using an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ (Hossain et al., 2006).

Glutathione peroxidase (EC: 1.11.1.9) activity: The reaction mixture contained of 100 mM K-P buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 0.12 mM NADPH, 2 mM GSH, 1 unit GR, 0.6 mM H_2O_2 (as a substrate), and 20 μL of sample solution. The oxidation of NADPH had been observed at 340 nm for 1 min and the activity was calculated using an extinction coefficient of 6.62 $mM^{-1}cm^{-1}$ (Hasanuzzaman et al., 2011).

Catalase (EC: 1.11.1.6) activity: Decrease of absorbance (by decomposition of H_2O_2) at 240 nm had been noticed for 1 min. The reaction had been started with enzyme extract; activity has been calculated using an extinction coefficient of 39.4 $M^{-1}cm^{-1}$ (Hasanuzzaman et al., 2011).

Glyoxalase I (EC: 4.4.1.5): The assay mixture contained 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulfate, 1.7 mM GSH, and 3.5 mM MG (final volume 700 μL). Adding MG the reaction had been started; the increase in absorbance was recorded at 240 nm for 1 min. Activity had been calculated using an extinction coefficient of 3.37 $mM^{-1}cm^{-1}$ (Hasanuzzaman et al., 2011).

Glyoxalase II (EC: 3.1.2.6): Formation of GSH was monitored for 1 min at 412 nm. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM DTNB, and 1 mM SLG (final volume of 1 mL). Reaction had been initiated by adding SLG; activity had been calculated using an extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ (Principato et al., 1987).

Lipoxygenase (EC 1.13.11.12): LOX activity was estimated monitoring the increase of absorbance at 234 nm using linoleic acid as a substrate. Activity had been calculated using an extinction coefficient of 25 $mM^{-1}cm^{-1}$ and expressed as units (1 nmol of substrate oxidized per min) mg^{-1} protein (Doderer et al., 1992).

Statistical Analysis

All data were subjected to analysis of variance (ANOVA). Mean differences had been compared by Tukey's HSD test using XLSTAT v. 2016.04.32525 software (Addinsoft, 2016). Differences at $P \leq 0.05$ were considered significant.

RESULTS

Production of ROS and Oxidative Stress

Cadmium stress imposition in the growing media caused oxidative damage in the seedlings. Membrane lipid peroxidation (increasing MDA levels) has been noticed in Cd-affected rapeseed seedlings (**Figure 1A**). Content of H_2O_2 increased by 37 and 60%, and activity of LOX increased by 62 and 145% under 0.5 and 1 mM $CdCl_2$ stresses (**Figures 1B,C**), respectively, as compared with control plants. All these were responsible for peroxidation of membrane lipid. Exogenous H_2O_2 application reduced H_2O_2 content and LOX activity which are corroborating with the reduction of MDA contents by 23 and 25% in mild and severe Cd stresses when compared to stress treatments only (**Figures 1A–C**).

Histochemical Detection of ROS in Rapeseed Leaves

Leaves were dipped into DAB and NBT solution to visualize the generation and spots of H_2O_2 and $O_2^{\bullet-}$, respectively. The leaves under Cd stress showed a high frequency of dark brown patches of H_2O_2 and deep blue spots of $O_2^{\bullet-}$ anions (**Figures 2A,B**). The spots were darker and larger in severe Cd stress, compared to the mild Cd stress. However, these spots of H_2O_2 and $O_2^{\bullet-}$ were somewhat reduced, compared to Cd stress alone when exogenous H_2O_2 was added with Cd stresses which are indicators for oxidative stress reduction (**Figures 2A,B**).

ASA-GSH Cycle

Ascorbate content decreased by 20 and 32%; in contrast, DHA content increased by 7 and 43% which resulted in 25 and 52% decrease of AsA/DHA ratio under mild and severe Cd stresses when compared to Cd untreated control. Increase of GSH pool and also with the high increase of GSSG resulted in decreased ratio of GSH/GSSG by 15 and 44%, respectively, under 0.5 and 1 mM $CdCl_2$ stress, respectively, compared to control. Exogenous H_2O_2 addition inverted the AsA-GSH pool by increasing AsA content by 32 and 30% (**Figure 3A**), increasing GSH content by 38 and 25% (**Figure 3D**), decreasing DHA content by 12 and 21% (**Figure 3B**), and decreasing GSSG content by 17 and 8% (**Figure 3E**), under mild and severe Cd stresses, respectively. Alteration of AsA and GSH contents by H_2O_2 pretreatment were vital in improving AsA/DHA (**Figure 3C**) and GSH/GSSG (**Figure 3F**) ratios, compared to Cd stress alone.

The enzymes [APX, MDHAR, DHAR, and GR] (**Figures 4A–D**) of AsA-GSH cycle responded differentially in Cd-exposed seedlings. APX activity increased, MDHAR and DHAR activities reduced with the increase of Cd dose, compared to control, whereas, GR activity increased under mild Cd stress but reduced under severe Cd stress in comparison to their respective control (**Figures 4A–D**). External application of H_2O_2 under Cd stress increased activities of APX (40 and 39%), DHAR (77 and 67%), and GR (36 and 79%), respectively, under mild and severe Cd stresses, respectively, in contrast to Cd stress alone (**Figures 4A–D**).

CAT, GPX, and GST Activities

Both levels of Cd stress-affected seedlings showed higher GST activity whereas GPX activity increased only under mild Cd stress level, but CAT activity decreased at both levels of Cd stresses when compared to control. Activity of GST upregulated by 115 and 145%, the activities of CAT reduced by 28 and 44% under mild and severe Cd stress, respectively; activity of GPX amplified by 23% under mild Cd stress but it decreased by 23% under severe

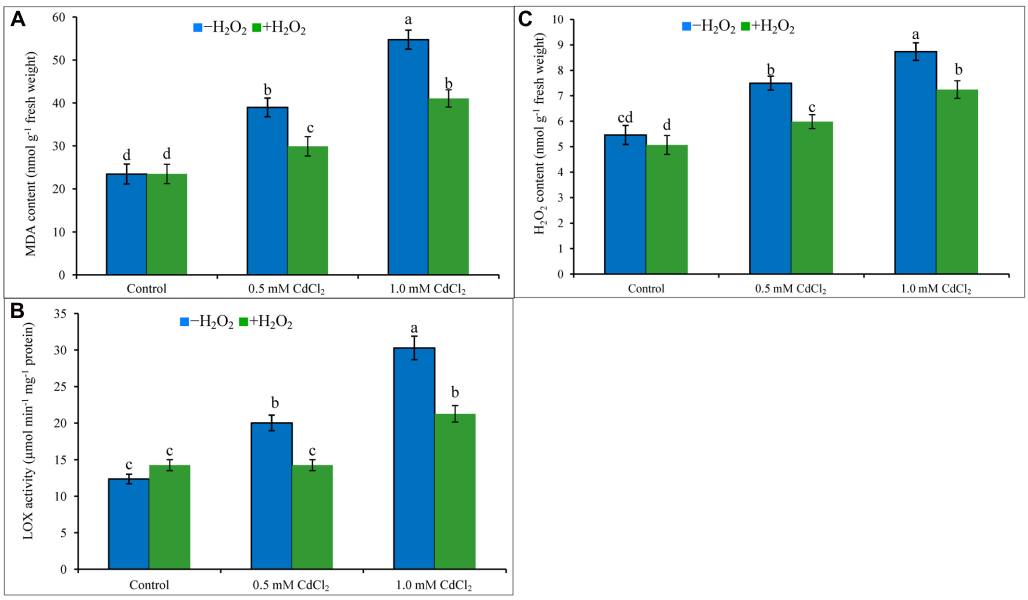

FIGURE 1 | Malondialdehyde content (A), H_2O_2 content **(B)**, and LOX activity **(C)** in rapeseed leaves induced by exogenous H_2O_2 under Cd stress. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

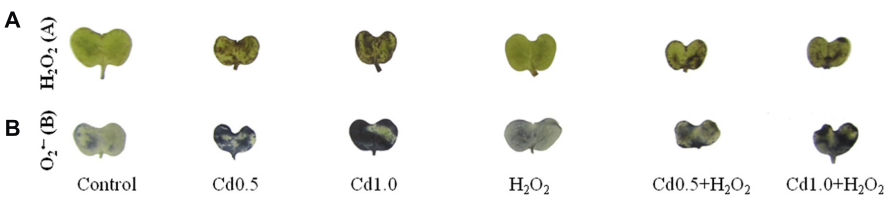

FIGURE 2 | 3,3-Diaminobenzidine staining (A) of H_2O_2 and NBT staining **(B)** of superoxide $O_2^{\bullet-}$ in rapeseed leaves induced by exogenous H_2O_2 under Cd stress.

Cd stress, compared to control (**Figures 5A–C**). Supplementation of H_2O_2 with Cd improved GST activities by 44 and 43%, and CAT activities by 79 and 47%, under mild and severe Cd stresses whereas augmented GPX activity by 40% under severe stress (**Figures 5A–C**), respectively, compared to Cd stress alone.

Glyoxalase System

Activities of Gly I and Gly II decreased due to exposure of Cd (**Figures 6A,B**). Their activities increased in both doses of Cd stress treatments supplemented with H_2O_2 except for Gly I activity at severe stress. The increase of Gly I activity under mild Cd stress was 35% after H_2O_2 supplementation, compared to Cd stress alone. Gly II activity increased by 47 and 55% in H_2O_2 added mild and severe Cd stresses, compared to Cd stress alone (**Figures 6A,B**).

DISCUSSION

Showing toxicity at higher concentration and acting as signaling molecule initiate, H_2O_2 plays a dual role and is considered as rival and comrade of stress tolerance development in plants. Due to dual roles of H_2O_2 and due to various unidentified roles of H_2O_2, recent research with H_2O_2 concentrate on diversified plausible mechanisms through which H_2O_2 is related to plant stress tolerance development. Present study has been executed to reveal the pivotal roles of H_2O_2 in relation to Cd stress tolerance in rapeseed.

The mechanism of Cd-induced oxidative stress is different from other stresses; Cd^{2+} cannot produce ROS directly as it through Fenton reaction or Haber Weiss reaction. Showing affinity to thiol Cd run downs GSH (Lopez et al., 2006). Cd enhances ROS production by weakening antioxidant defense mechanism (Srivastava et al., 2004; Gill and Tuteja, 2010), distressing photosystem II activity (Sigfridsson et al., 2004), disturbing functioning of vital enzymes (Dong et al., 2006). Cd displaces iron (Fe) from proteins and increases free Fe that is responsible for ROS generation. Cd also increases ROS production distorting mitochondrial function (Dorta et al., 2003). Spots of H_2O_2 and $O_2^{\bullet-}$ in leaves of rapeseed and the contents of H_2O_2 and MDA have been increased considerably in Cd affected rapeseed plants clearly indicating oxidative damage corroborating the results of previous studies (Dong et al.,

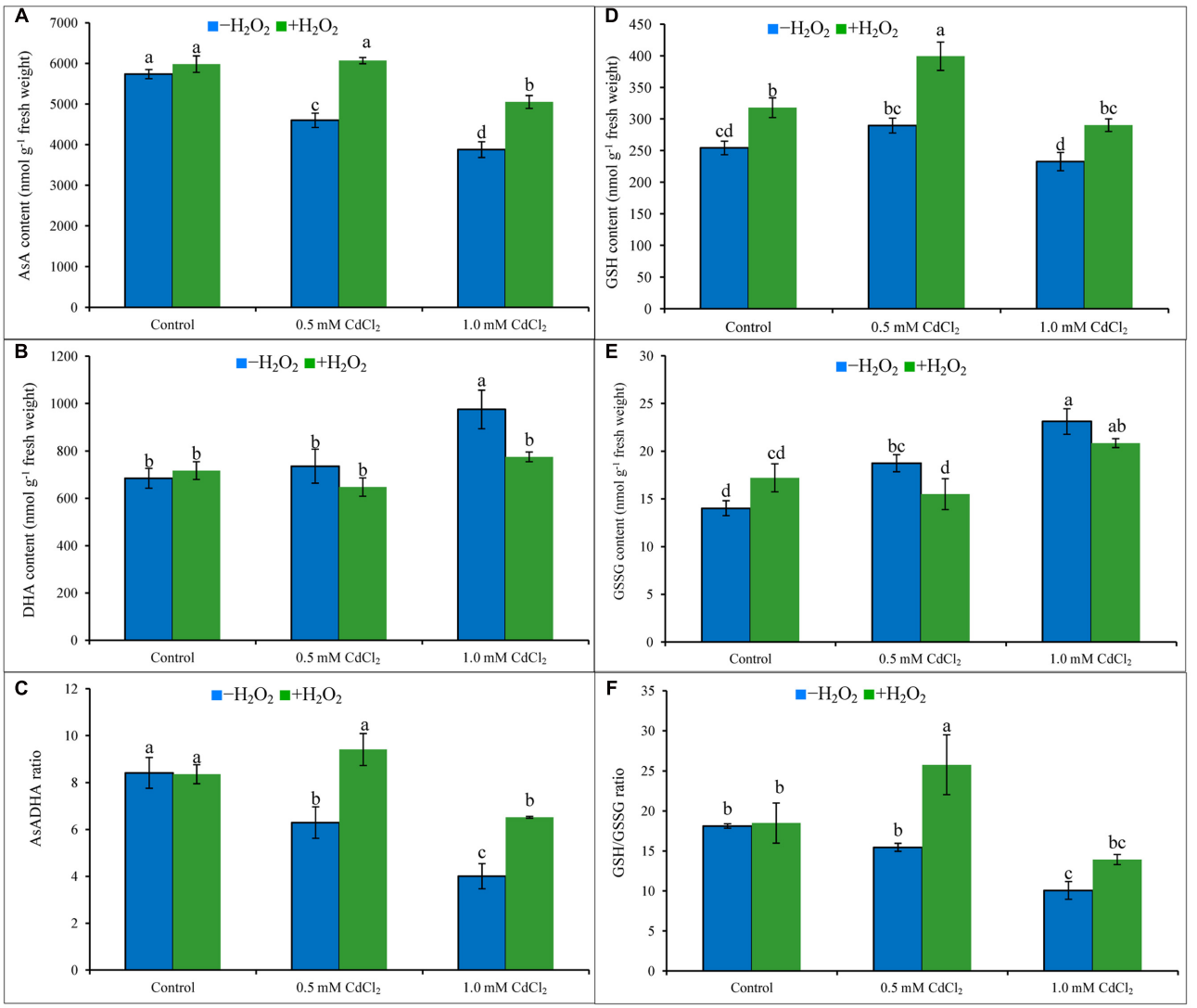

FIGURE 3 | Ascorbate (AsA) content (A), DHA content **(B)**, AsA/DHA ratio **(C)**, GSH content **(D)**, GSSG content **(E)**, and GSH/GSSG ratio **(F)** in rapeseed leaves induced by exogenous H_2O_2 under Cd stress. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

2006; Hu et al., 2009; Hasanuzzaman et al., 2012a). H_2O_2 pretreatment reduced oxidative damage by decreasing the spots of $O_2^{\bullet-}$ and H_2O_2 and reducing the amount of H_2O_2 and MDA contents against Cd toxicity (Hu et al., 2009), reducing contents of MDA and $O_2^{\bullet-}$ in salinity affected wheat plants (Li et al., 2011), decreasing $O_2^{\bullet-}$, H_2O_2 and MDA in chill affected cucumber seedlings (Zhang et al., 2011). The results of these previous reports indicate the decisive functions of H_2O_2 in reducing oxidative stress. At low concentration, H_2O_2 can as signaling molecule which modulates various genes related to stress defense mechanism. H_2O_2 implicated NO-mediated ABA-induced activation of mitogen-activated protein (MAP) kinase cascade which modulated antioxidant defense mechanism maize leaves. H_2O_2 can modulate NO and NO itself is an ROS scavenger (Zhang et al., 2007). In present study, the advantageous roles of H_2O_2 have been presented in later section where application of very low concentration exogenous H_2O_2 pretreatment induced and enhanced the antioxidant defense system components which in turn helped in decreasing the endogenous contents of ROS including H_2O_2 and $O_2^{\bullet-}$ and in decreasing the oxidative damage which is parallel with the results of the previous findings (Zhang et al., 2007, 2011). Both AsA and GSH presenting in chloroplast, cytoplasm, apoplast, mitochondria, peroxisome effectively scavenge H_2O_2. CAT, APX, GPX, and GST directly catalyze the reactions of H_2O_2 scavenging. Exogenous low dose of H_2O_2 in the present study enhanced the activities of these enzymes and increased the contents of AsA and GSH of Cd affected rapeseed seedlings which are directly related to H_2O_2 scavenging process and that is why H_2O_2 pretreatment decreased the endogenous H_2O_2 levels and subsequent oxidative damage of

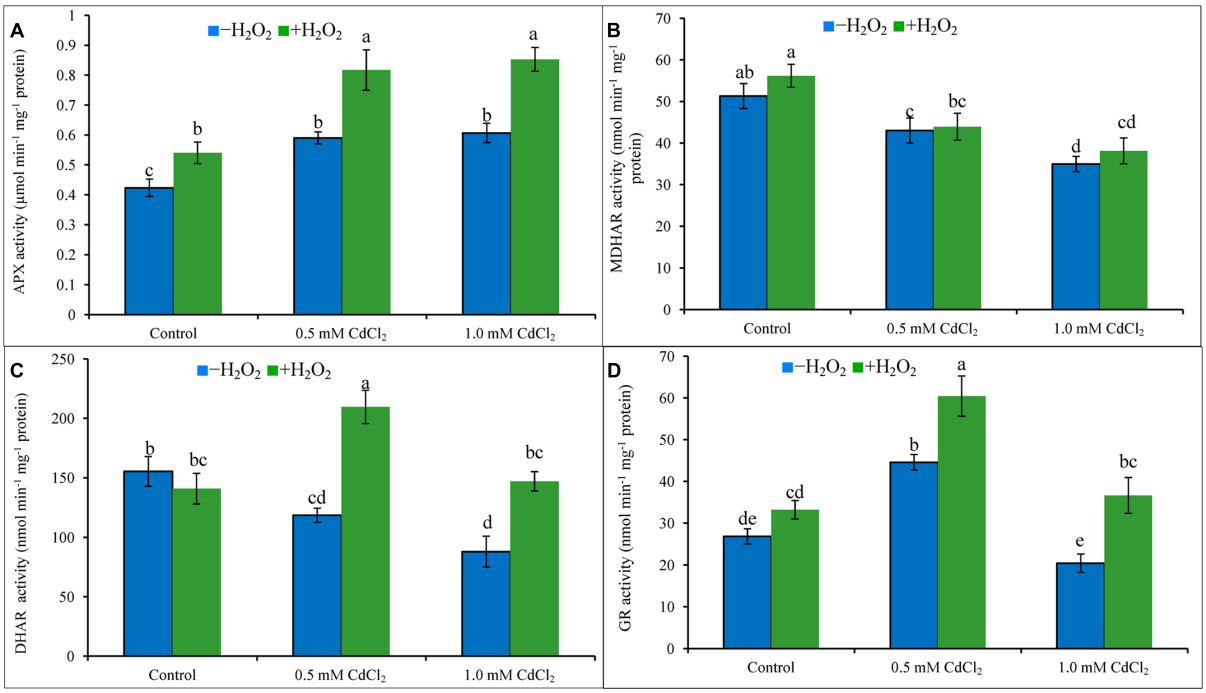

FIGURE 4 | Activities of AsA-GSH cycle enzymes, APX (A), MDHAR **(B)**, DHAR **(C)**, and GR **(D)** in rapeseed leaves induced by exogenous H_2O_2 under Cd stress. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

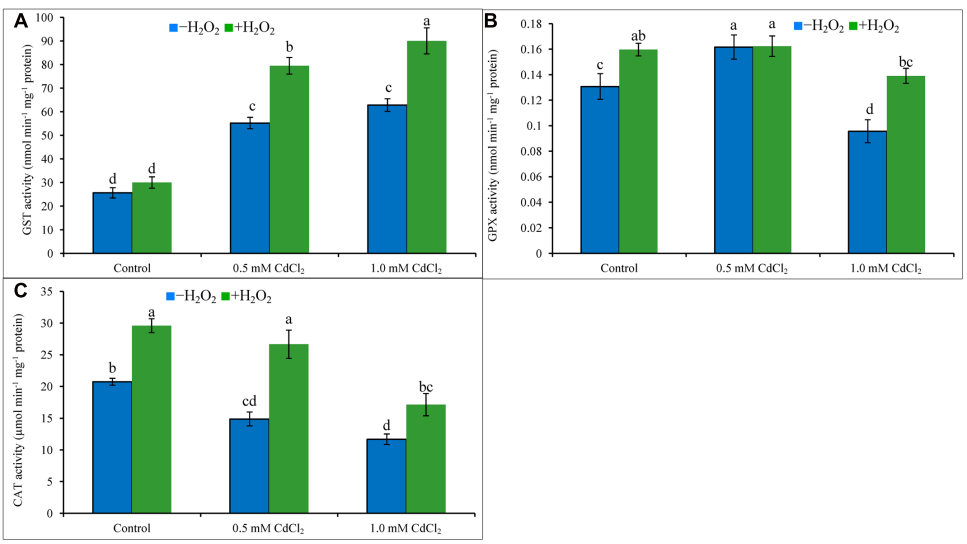

FIGURE 5 | Activities of GPX (A), GST **(B)**, and CAT **(C)** in rapeseed leaves induced by exogenous H_2O_2 under Cd stress. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

Cd affected seedlings (Mittler, 2002; Blokhina et al., 2003; Ashraf, 2009; Gill and Tuteja, 2010).

Ascorbate is water-soluble non-enzymatic antioxidant in cell decreasing oxidative stress scavenging $O_2^{\bullet-}$ and $OH^{\bullet}$ (Gill and Tuteja, 2010). In rapeseed seedlings of present study, AsA level reduced and DHA level increased (because AsA is oxidized to DHA after scavenging ROS) due to reduced MDHAR and DHAR activities (which are AsA recycling enzymes) which decreased the AsA/DHA ratio and increased oxidative stress (Chao et al., 2010). APX activity upregulated due to Cd exposure, which is correlated to the reduced AsA content. H_2O_2 pretreatment followed by Cd exposure upregulated APX, MDHAR and DHAR

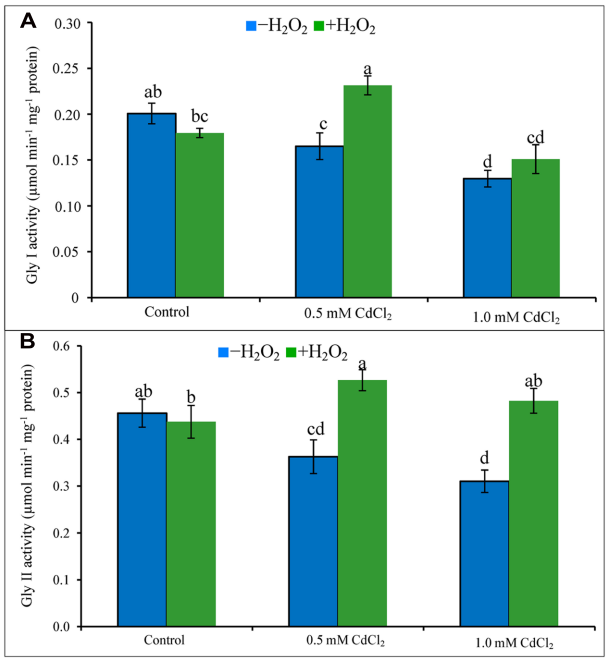

FIGURE 6 | Activities of Gly I (A) and Gly II **(B)** in rapeseed leaves induced by exogenous H_2O_2 under Cd stress. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

activities in the seedlings which restored AsA and decreased oxidative stress which is supported by previous findings (Hu et al., 2009; Li et al., 2011; Zhang et al., 2011). In rapeseed seedlings, increased levels of GSH and GSSG but decreased GSH/GSSG ratio have been noticed in exposure to Cd which are supported by previous studies (Molina et al., 2008; Hu et al., 2009; Hasanuzzaman et al., 2012a). GSH is the thiol group of non-enzymatic antioxidant showing an imperative role in the stress signal, adaptation and defense mechanism of plants (Noctor et al., 2012). GSSG is recycled to GSH involving the GR activity (Gill and Tuteja, 2010). Increased GSH content after H_2O_2 application was found beneficial under Cd stress (Chao et al., 2009). The application of H_2O_2 upregulated AsA and GSH levels and improved CAT, POD, SOD, GPX, GR, MDHAR, and DHAR metabolism as reported in Al affected wheat seedlings (Xu et al., 2010). Seedlings pretreated with H_2O_2 increased GR activity (which recycles GSSG to GSH) which resulted in decreased GSSG level and increased GSH content which increased GSH/GSSG ratio in Cd affected rapeseed seedlings which is supported by previous findings (Hu et al., 2009; Li et al., 2011; Zhang et al., 2011).

The multifunctional isoenzymes GSTs are vital antioxidant enzymes, involved xenobiotic and toxic compound detoxification process (Polidoros and Scandalios, 1999). In the present investigation, GST activity and GSH amplified due to Cd exposure which were also observed in other studies (Hu et al., 2009; Hasanuzzaman et al., 2012a). Nonetheless, a further increase of GST activity and GSH content was noticed in H_2O_2 pretreated rapeseed seedlings under Cd stress which reduced adverse effects of Cd on physiology and growth which is similar to the findings of Hu et al. (2009) in rice. Increased Cd sequestration by H_2O_2 pretreatment in rice roots is an indication of crucial roles of H_2O_2 to further decline of Cd translocation to shoot (Hu et al., 2009). Cd stress reduced CAT and GPX activities in rapeseed seedlings that are correlated to a generation of high H_2O_2 which is comparable with previous findings (Hu et al., 2009; Hasanuzzaman et al., 2012a). The Cd has an affinity to proteins or –SH compounds and other side chains, Cd disturbs protein and enzymes synthesis which impair enzymatic activity (Sanitá di Toppi and Gabbrielli, 1999). CAT and GPX activities were restored and increased in H_2O_2 pretreated Cd-stressed seedlings which played vital roles in reducing the H_2O_2 level in Cd affected seedlings. Similar roles of H_2O_2 were observed in Cd affected rice seedlings (Hu et al., 2009), cucumber plants subjected to low light stress (Zhang et al., 2011) and in salt affected rice seedlings (Li et al., 2011).

Like other abiotic stresses, MG is overproduced within the plants under Cd stress (Hasanuzzaman et al., 2012a; Kaur et al., 2015; Nahar et al., 2015). Cd decreased both the activities of Gly I and Gly II that indicated the reduced MG detoxification via the glyoxalase system (Nahar et al., 2015). In this study, rapeseed seedlings exposed to Cd decreased Gly I and Gly II activities but pretreatment with H_2O_2 increased Gly I and Gly II activities and GSH content indicating the imperative roles of H_2O_2 in MG detoxification which are in the same line with the previous findings (Hasanuzzaman et al., 2012a; Nahar et al., 2016a,b).

CONCLUSION

In this study, we provided evidence for a specific pattern of ROS generation (H_2O_2 and $O_2^{\bullet-}$) and oxidative damage (MDA content) with the raise of Cd dose. Exogenous H_2O_2 treatment increased the amount of the most important ROS scavenging molecules AsA and GSH and increasing the antioxidant enzyme activities which enhanced ROS scavenging process. The Gly I and Gly II activities and content of GSH increased after H_2O_2 pretreatment indicating the roles of exogenous H_2O_2 in MG detoxification process. In contrast to the evidence of exogenous H_2O_2-induced ROS and MG detoxification in the present study, a number of unanswered questions still remain unclear. Why and how does H_2O_2 induce production of antioxidant molecules (AsA and GSH) and activities antioxidant enzymes? Previous reports support the notion that H_2O_2 induced signaling is involved with phytohormones and signaling molecules ABA, SA, JA (jasmonic acid), GA (gibberallic acid), ethylene, NO, Ca^{2+}-mediated development of abiotic stress tolerances in plant (Mittler et al., 2004; Jubany-Mari et al., 2009; Chen et al., 2012; Guo et al., 2014; Reczek and Chandel, 2015; Saxena et al., 2016). In relation to the findings of present study, new questions arise: is there any signaling function of H_2O_2 in regulating the biosynthesis or degradation/metabolism of antioxidants

components or, other hormones or signaling molecules affecting these processes? The possible mechanisms and signaling action of H_2O_2 in these aspects should be further elucidated.

AUTHOR CONTRIBUTIONS

MH, MF, and KN conceived and designed the experiments; MH and KN performed the experiments; HA and BR analyzed the data; MF contributed reagents/materials/analysis tools; MH, KN, SG, HA, and BR wrote the manuscript. BR edited the manuscript. All authors read and approved the final manuscript.

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