INTERPLAY BETWEEN NO SIGNALING, ROS, AND THE ANTIOXIDANT SYSTEM IN PLANTS

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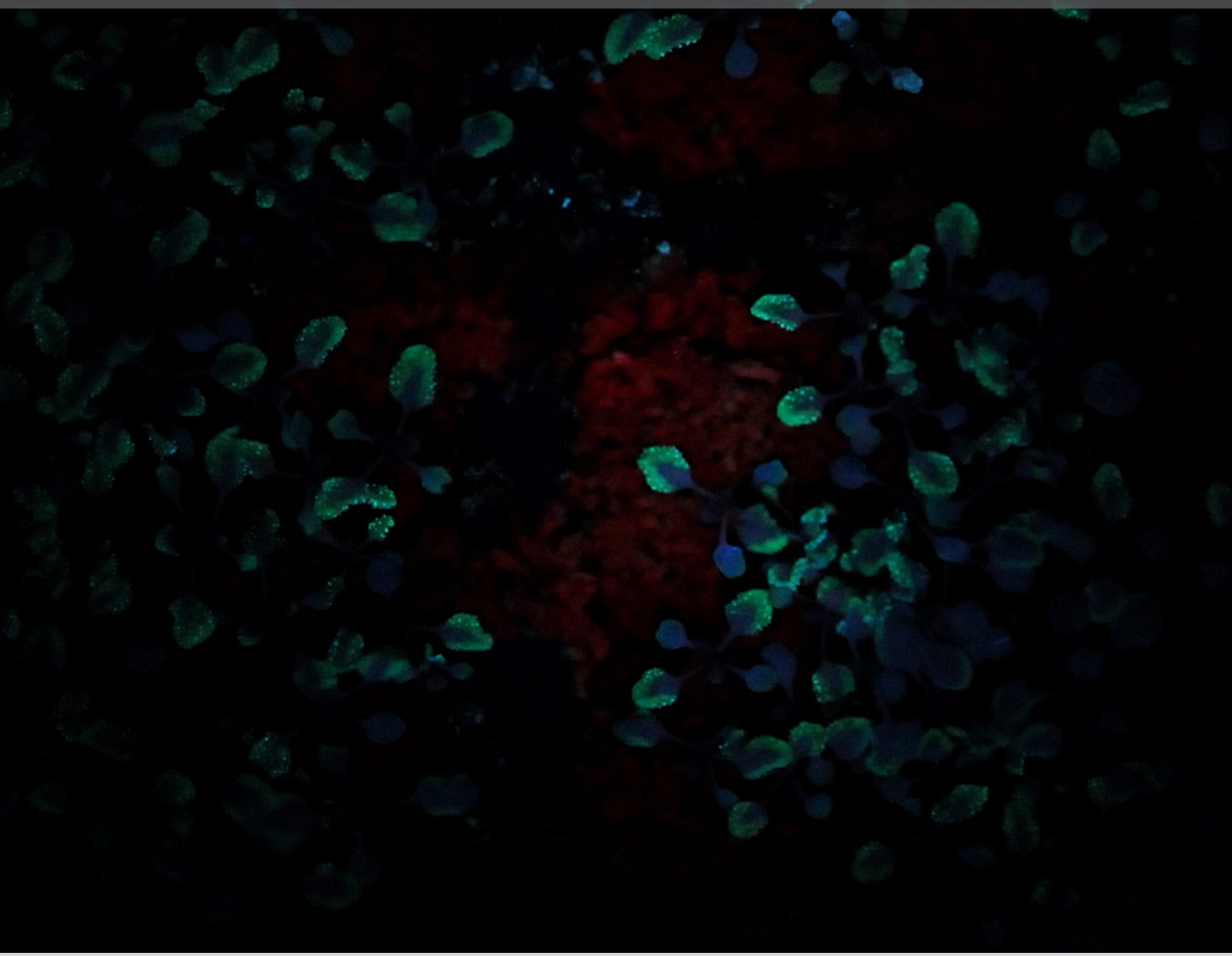

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INTERPLAY BETWEEN NO SIGNALING, ROS, AND THE ANTIOXIDANT SYSTEM IN PLANTS

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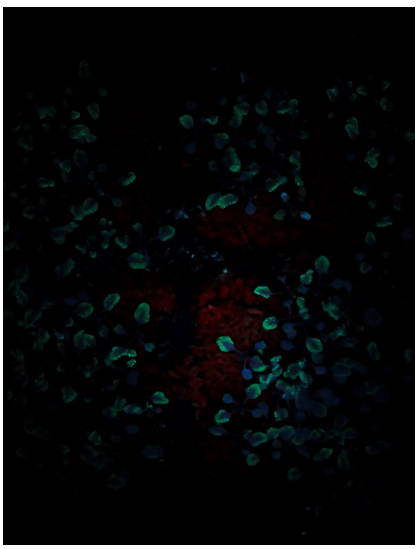

Accumulation of fluorescent secondary metabolites in Arabidopsis plants undergoing NO- and H_2O_2-dependent cell death as visualized under UV light.
Image by Frank Gaupels

Over the last decades, nitric oxide (NO) has emerged as an essential player in redox signaling. Reactive oxygen species (ROS) also act as signals throughout all stages of plant life. Because they are potentially harmful for cellular integrity, ROS and NO levels must be tightly controlled, especially by the classical antioxidant system and additional redox-active metabolites and proteins. Recent work provided evidence that NO and ROS influence each other's biosynthesis and removal. Moreover, novel signaling molecules resulting from the chemical reaction between NO, ROS and plant metabolites have been highlighted, including N_2O_3, $ONOO^-$, NO_2, S-nitrosoglutathione and 8-NO_2 cGMP. They are involved in diverse plant physiological processes, the best characterized being stomata regulation and stress defense. Taken together, these new data demonstrate the complex interactions between NO, ROS signaling and the antioxidant system. This Frontiers in Plant Science Research Topic aims to provide an updated and complete overview of this important and rapidly expanding area through original article and detailed reviews.

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NO, ROS and Antioxidant System Signaling in Plant

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EDITORIAL
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Editorial: Interplay between NO Signaling, ROS, and the Antioxidant System in Plants

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Keywords: reactive oxygen species, nitric oxide, antioxidant system, plant defense, biotic and abiotic stress, plant development

Editorial on the Research Topic

Interplay between NO Signaling, ROS, and the Antioxidant System in Plants

Over the last decades, reactive oxygen and nitrogen species (ROS and RNS), and particularly nitric oxide (NO), have been linked to a wide variety of physiological processes in plants, ranging from the control of developmental processes to the regulation of plant responses against biotic or abiotic stress. In addition to the regulation of ROS and RNS biosynthesis, the exquisite modulation of their activities is also under the control of a complex network of regulatory enzymes and compounds constituting the antioxidant system. The Research Topic on the *Interplay between NO signaling, ROS and the antioxidant system in plants* aims to provide an up-to-date view of this increasingly important area through both original articles and detailed reviews.

NO and ROS signaling pathways are key regulators of plant development. In this context, Ma et al. studied the role of NO, ROS, and the antioxidant system on the germination of barley seeds. They found that the turnover of NO via the action of phytoglobin and S-nitrosoglutathione reductase and also its interaction with ROS contributed to the alleviation of seed dormancy. The impact of oxidative stress on plant development is also outlined in the mini-review presented by Frank and Ernst where they summarize the current knowledge about the effect of the air pollutants NO_2 and ozone on pollen allergenicity. The authors report that both NO_2 and ozone result in increased pollen allergenicity, in a dose-dependent and species-specific manner. Further, corroborating the pivotal role of ROS in developmental processes, Jiménez-Quesada et al. summarize in an extensive review the importance of NADPH oxidase activity on sexual plant reproduction mechanisms.

The interplay between NO and ROS in the early development of the symbiotic interaction between plants and rhizobium partners is also reviewed in this Topic by Damiani et al. They describe how the spatiotemporal accumulation of these compounds is tightly regulated for the successful establishment of the symbiosis, both in the plant host and the bacteria symbiont.

The role of NO, ROS, and the antioxidant system during plant/pathogen interactions is also addressed in this Topic. Thalineau et al. studied their role the infection of *Medicago truncatula* by *Aphanomyces euteiches*. They demonstrate that NO homeostasis impacts nitrate reductase activity and therefore N nutrition. They propose a link between NO/ROS homeostasis, N nutrition and plant immunity. Gaupels et al. explored the long distance signaling induced in the extrafascicular phloem of pumpkins during systemic wound responses. They report that a decrease in the antioxidant system capacity correlates with an increase in NO/ROS content. Sivakumaran et al.

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reveal an antagonistic role of abscisic acid on NO/ROS generation in tomato following challenge with *Botrytis cinerea*. They propose this mechanism might be a plant defense suppressing strategy exhibited by the pathogen enabling the development of a compatible interaction. The significance of ROS and NO during biotic interactions from the pathogen perspective is also developed in this Topic. Arasimowicz-Jelonek and Floryszak-Wieczorek review this question focusing on the specific role of NO in pathogenic fungi and oomycetes. In an original work, Yin et al. show that both NO and ROS are crucial players in the germination of urediniospores of *Puccinia striiformis* f. sp. *tritici*, the agent responsible for stripe rust in wheat.

Another important feature of this Topic focuses on the plant responses to abiotic stresses. Farnese et al. provide a comprehensive overview of NO, ROS, and the antioxidant system in plant adaptive responses to abiotic stress, emphasizing their crosstalk and specificities depending on the given stress. Fatma et al. underline the effect of NO and sulfur on photosynthetic performance of mustard plants exposed to salt stress, demonstrating that the combined application of NO and S provides the best protection of photosynthetic machinery. In an original article, Lytvyn et al. report the link between inositol and NO signaling in the development of oxidative stress following ultraviolet-B irradiance in *Arabidopsis thaliana*. Also, Wu et al. describe the relation between NO and heme oxidase 1 in germinating rice aleurone layer subjected to drought stress. They describe how the mutual induction of these two factors affects gibberellin-induced programmed cell death following germination. In addition to these original data, Molassiotis et al. discuss in a mini-review the mechanism associated with NO and H_2O_2 priming of citrus defense responses prior to abiotic stress, whereas Gupta and Igamberdiev review the crucial role of mitochondria in modulating RNS and ROS signaling to promote plant survival on exposure to hypoxia or anoxia.

The better understanding of the NO- and ROS-dependent biochemical mechanisms themselves is another aspect developed in this topic. Krasuska et al. analyzed the mode of action of the tomato root growth inhibitor canavanine (CAN), a non-proteogenic amino acid and a structural analog of arginine, used as a mammalian nitric oxide synthase inhibitor. They conclude that, despite decreasing NO levels, the rapid response of plants to CAN exposure probably results from an increase in carbonylated proteins following ROS production. The post-translational modification of proteins is also the main subject reviewed by Begara-Morales et al. These authors detail our present knowledge regarding the NO modification of enzymes integral to the plant antioxidant system, with a particular focus on the components of the ascorbate-glutathione cycle. Further, Romero-Puertas and Sandalio discuss how NO might regulate its own levels and that of its ROS partners. They discuss the important factors integral to the fine tuning existing between NO and ROS signaling. Finally, Gross and Durner reviewed recent research data pertaining to the NO-3′,5′-cyclic guanosine monophosphate (cGMP) dependent pathway in plants. While NO mediated cGMP signaling is well described in mammals, this system is not well defined in plants. These authors discuss the current situation concerning the identification of enzymes involved in such a pathway in higher plants.

The wide variety of the work reported here testifies to the importance of NO, ROS, and the antioxidant system to plant physiology, and highlights the complexity of their interplay. We hope that this Topic will provide an update of this important and rapidly expanding area and also offer a primer to drive future exciting lines of research enquiry.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Nitric Oxide and Reactive Oxygen Species Mediate Metabolic Changes in Barley Seed Embryo during Germination

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The levels of nitric oxide (NO) and reactive oxygen species (ROS), ATP/ADP ratios, reduction levels of ascorbate and glutathione, expression of the genes encoding proteins involved in metabolism of NO and activities of the enzymes involved in fermentation and in metabolism of NO and ROS were studied in the embryos of germinating seeds of two barley (*Hordeum vulgare* L.) cultivars differing in dormancy level. The level of NO production continuously increased after imbibition while the level of nitrosylated SH-groups in proteins increased. This corresponded to the decrease of free SH-groups in proteins. At early stage of germination (0–48 h post imbibition) the genes encoding class 1 phytoglobin (the protein scavenging NO) and *S*-nitrosoglutathione reductase (scavenging *S*-nitrosoglutathione) were markedly expressed. More dormant cultivar exhibited lower ATP/ADP and ascorbate/dehydroascorbate ratios and lower lactate and alcohol dehydrogenase activities, while the production of NO and nitrosylation of proteins was higher as compared to the non-dormant cultivar. The obtained data indicate that at the onset of germination NO is actively generated causing nitrosylation of SH-groups and a switch from respiration to fermentation. After radicle protrusion the metabolism changes in a more reducing type as recorded by ratio of reduced and oxidized glutathione and ascorbate. The turnover of NO by the scavenging systems (phytoglobin, *S*-nitrosoglutathione reductase and interaction with ROS) might contribute to the maintenance of redox and energy balance of germinating seeds and lead to alleviation of dormancy.

Keywords: barley (*Hordeum vulgare* L.), dormancy, germination, nitric oxide, reactive nitrogen species, reactive oxygen species

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INTRODUCTION

The early stage of seed germination starting from the uptake of water by dry seeds is a challenge for plants, in particular because seed coat represents a robust barrier for oxygen (Bewley, 1997). Only after radicle protrusion the mobilization of storage reserves becomes not significantly limited by oxygen supply (Taylorson and Hendricks, 1977; Bewley and Black, 1994; Bewley, 1997). As a consequence, after imbibition seeds develop anaerobic conditions limiting oxidative respiration

(reviewed in Bykova et al., 2015). Despite this, seed ATP levels and energy charge remain high in early germination (Duff et al., 1998; Logan et al., 2001; Benamar et al., 2003). The increase of reduction level of NAD, NADP, and other electron transferring compounds upon oxygen depletion immediately after imbibition results in the formation of reactive species of oxygen (ROS) and of nitrogen (RNS) (Bethke, 2009).

It has been suggested that generation of nitric oxide (NO) under seed coat is increased upon depletion of oxygen representing a mechanism of supporting redox and energy balance in germinating seeds together with activation of fermentation processes (Dębska et al., 2013). In germination, after the initial depletion of oxygen within several hours, the transition to the mainly anaerobic step takes place, characterized by NO production. These phases correspond to the stages I and II described by Bewley and Black (1994). While the generation of NO is an unavoidable consequence of low oxygen and high reduction level of electron transferring compounds (Igamberdiev and Hill, 2004), the turnover of NO that leads to balancing of redox and energy levels is achieved via the systems participating in scavenging of RNS such as class 1 plant hemoglobin (phytoglobin; Igamberdiev et al., 2006) and S-nitrosoglutathione reductase, which detoxifies nitrosylated glutathione returning it to the active glutathione pool and represents one of the reactions catalyzed by the class III alcohol dehydrogenase (Corpas et al., 2013). This results in the oxidation of NAD(P)H and possibly in the support of ATP formation by mitochondria (Stoimenova et al., 2007). However, the particular aspects of NO and RNS metabolism in germinating seeds are not investigated in detail and need further clarification.

It has been suggested that NO and ROS are crucial for dormancy breaking, while generation of NO under seed coat is increased upon depletion of oxygen representing a mechanism of supporting redox and energy balance in germinating seeds together with the activation of fermentation processes (Bethke et al., 2004, 2007; Igamberdiev and Hill, 2004). While ROS formation starts upon imbibition, the NO production is triggered by the progressing depletion of oxygen under seed coat upon germination. NO nitrosylates proteins and glutathione, while ROS can interact with NO and facilitate its scavenging (Wulff et al., 2009). The interplay between NO and ROS is important for understanding how the seed breaks dormancy and maintains its energy status during germination (Bykova et al., 2015).

In this paper we have presented a wide-range study of the development of oxidative metabolism in embryo during the first 2 days of germination of the cereal (barley) seeds of two cultivars with different germination rates. The main part of embryo in early germination is scutellum which supplies intermediates for carbohydrate and amino acid synthesis and contributes to acidification of endosperm. We have traced the development of production of NO, ROS and their scavenging systems in the course of germination of two barley cultivars differing in dormancy level. It is concluded that NO production results in the increase of protein nitrosylation. The mechanisms involved in scavenging of NO and RNS include the expression of phytoglobin and GSNO-reductase, as well as the interaction of NO and superoxide anion with the following degradation of peroxynitrite. The role of NO turnover during the early stage of plant development is discussed in relation to the maintenance of redox and energy levels in cells, which supports the development of germinating seeds and facilitates the efficiency of responses to biotic and abiotic stress factors in the course of germination process and dormancy breaking.

MATERIALS AND METHODS

Barley Seeds

Seeds of two barley (*Hordeum vulgare* L.) cultivars, Harrington (non-dormant, 96% of seeds germinate after 24 h) and Sundre [more dormant, its germination resistance is about 1.2 days determined by the method of Gordon (1971)] were soaked with sterile deionized water from 0 (dry seeds) to 48 h on filter paper in Petri dishes. Embryos (usually 100 mg) were isolated and ground in liquid nitrogen with mortar and pestle. The Sundre embryos analyzed from 0 to 9 h after imbibition could not be distinguished into dormant and non-dormant and therefore represented a mixture of seeds with different germination potential.

Measurement of Protein Concentration

The method of measuring concentration of proteins was based on standard protocol of Bradford reagent (Sigma–Aldrich). Bovine serum albumin was used as a standard.

ATP and ADP Measurement

Extraction of ATP and ADP was conducted according to Joshi et al. (1989) and Yuroff et al. (2003). The tissue powder was lysed in ice-cold 2.4 M perchloric acid (1 ml to 25 mg) for 60 min on ice, centrifuged 5 min at 20,000 × *g*, and supernatant (0.5 ml) was neutralized by 4 M KOH. Quantification of ATP in the neutralized solution was performed by chemiluminescent analysis using the protocol of ATP Detection Kit (Thermo Fisher Scientific). Content of ADP was determined using EnzyLightTM ADP Assay Kit (EADP-100, BioAssay Systems). Fine tissue powder (25 mg) was homogenized in 200 μl of ice-cold 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 12,000 × *g* for 5 min. Supernatant was used for ADP analysis, followed the standard protocol of the kit.

Measurement of NO Content

Preparation of hemoglobin (Hb) solution and NO measurement were conducted according to Murphy and Noack (1994). Hb (50 mg) was dissolved in 10 ml of 50 mM Tris-HCl, pH 7.0. Sodium dithionite ($Na_2S_2O_4$, 15 mg) was added directly into fresh Hb solution (which contained mainly MetHb) and reacted for 3 min to reduce MetHb (Fe^{3+}) to Hb (Fe^{2+}), which immediately became HbO_2. The prepared HbO_2 solution was desalted through PD-10 column (GE Healthcare Life Sciences). The final concentration of HbO_2 was determined by spectrophotometry at 415 nm ($\varepsilon = 131\ mM^{-1}\ cm^{-1}$), adjusted to ∼10 μM and stored in dim light.

Nitric oxide was extracted from tissue powder with 50 mM Tris-HCl buffer, pH 7.0, containing 0.6% (w/v) PVP at 4°C. Supernatant was collected after centrifugation for 10 min at

15000 × *g*. To 0.5 ml of supernatant 12.5 μl superoxide dismutase (SOD; 4,000 U/ml) and 12.5 μl catalase (10,000 U/ml) was added and incubated for 2 min at room temperature to remove ROS. A volume of 0.475 ml of the desalted HbO_2 solution was added and the tubes were incubated for 5 min. Absorbance at 401 and 421 nm was recorded to calculate the concentration of NO in tissue using the extinction coefficient $\varepsilon = 77\ mM^{-1}\ cm^{-1}$.

It is recommended to use the two methods for measuring NO (Gupta and Igamberdiev, 2013). The measurement of NO content by the hemoglobin method was in agreement with the method using chemiluminescent NO analyzer CLD 88 p (Eco-Physics, Dürnten, Switzerland; not shown). However, it was not possible to compare these two methods before radicle protrusion when NO is trapped inside the seed coat, therefore we present here the data obtained by the hemoglobin method.

Measurement of Protein SH-Groups and *S*-Nitrosylation

Measurement of protein *S*-nitrosylation was conducted according to Jaffrey et al. (2001) by reduction of R-SNO to R-SH in the presence of ascorbate followed by the assay of free thiol groups using 5,5′-dithiol-bis (2-nitrobenzoic acid; DTNB; Riddles et al., 1983). Proteins were extracted from tissue powder with 50 mM HEPES, pH 8.0, containing 1 mM EDTA, 0.1 mM neocuproine, 0.2% (w/v) SDS and 0.5% (w/v) CHAPS. The samples were centrifuged at 4°C for 10 min at 15,000 × *g* and proteins were precipitated from supernatant by two volumes of acetone (−20°C) overnight. After centrifuging at 4°C and 15,000 × *g* for 10 min, protein precipitate was washed four times with chilled 70% acetone. The protein samples were re-suspended in the same volume of the extraction buffer. The clear protein solution was collected to measure the quantity of R-SNO. Ascorbate (50 μl of 100 mM solution) was added in 0.9 ml samples, the same volume of H_2O was added to control. After incubation for 1 h at 25°C, 50 μl of 10 mM DTNB in 75 mM phosphate buffer, pH 7, was added, followed by measurement of absorbance at 412 nm against control samples. The mixture of ascorbate and DTNB in extraction buffer, and DTNB in the same buffer were set up as blank for treatment and control groups, respectively. The difference of R-SH quantity between sample and control groups was used to calculate quantity of R-SNO. Namely, the quantity of R-SH generated by ascorbate treatment corresponded to that of R-SNO in proteins. The measurement of free SH-groups in proteins was conducted without ascorbate treatment.

Measurement of Superoxide Anion and Hydrogen Peroxide

To extract $O_2^{\bullet-}$, the fine powder of fresh tissue was homogenized in 8 M KOH and centrifuged for 10 min at 15000 *g* at 4°C. The superoxide anion level was measured in supernatant by the method modified from Sun and Trumpower (2003) and Dahlgren et al. (2007) at 550 nm by reduction of cytochrome *c*. Cytochrome *c* (0.1 ml, 5 mg ml^{-1}) was dissolved in 1.397 ml of 0.2 M potassium phosphate buffer, pH 8.6, and 3 μl extract solution was added finally, homogenized immediately and incubated for 15 min at 25°C. In the reference set 50 units of SOD and 100 units of catalase were added to the solution. The value of $\varepsilon = 21.5\ mM^{-1}\ cm^{-1}$ for the reduced cytochrome c (Fe^{2+}) was used, and based on a one-to-one molar stoichiometry between $O_2^{\bullet-}$ produced and cytochrome *c* molecules reduced, the actual amount of $O_2^{\bullet-}$ produced was quantified.

Concentration of hydrogen peroxide was measured according to the method of Lu et al. (2009). Fine powder of isolated scutella was homogenized in 6% trichloroacetic acid (TCA) for 30 min at 4°C, centrifuged at 15000 × *g* for 10 min, and then insoluble polyvinylpyrrolidone (PVPP; 50 mg/ml) was added. The samples were centrifuged at 15000 × *g* for additional 3 min. The extract was diluted 1000 fold with 0.1 M carbonate buffer (pH 10.2). The preparation of reagents followed the method of Pérez and Rubio (2006) and Lu et al. (2009). Ten ml of 6.5 mM luminol and 2 ml of 3 mM $CoCl_2$ in 0.1 M sodium carbonate buffer (pH 10.2) were mixed, diluted to 100 ml in the same buffer and stored for at least 1 h in dark. The solution was further diluted 10 times in the same buffer and stored at 4°C in dark overnight before use. The 40 μl sample was mixed with 10 μl of the sodium carbonate buffer and the mixture was incubated at 30°C for 15 min. Catalase (500 units) was added and incubated at the same condition as a control. Ten μl of each sample and 200 μl of reaction reagent were added into 5 ml SARSTEDT tubes to measure chemiluminescence (CL) with FB 12 Luminometer (Berthold Detection Systems GmbH, Germany). The difference of CL response between each treatment and corresponding background was considered as CL specific for H_2O_2 in samples. The amount of H_2O_2 produced per gram tissue was calculated using the standard curve.

Expression of the Genes Encoding *S*-Nitrosoglutathione Reductase and Class 1 Phytoglobin

Primers of *ADH3* (GenBank: X12734.1), *Hb* (GenBank: U94968.1), and *Mub1* (GenBank: M60175.1) for Real-Time Polymerase Chain Reaction (RT PCR) were designed using NCBI/Primer-BLAST according to known cDNA sequence of the *ADH3* and *Hb* genes encoding correspondingly GSNOR (which is a class III alcohol dehydrogenase) and class 1 phytoglobin, where *Mub1* was set up as a reference gene. Specific primers of *ADH3*, *Hb,* and *Mub1* were: ADH3-forward: 5′ - GTCTCTCAACTGG ACTTGGTG - 3′ and ADH3-reverse: 5′ - CTTAGCTTGTTCGT ATTTTGCAGG - 3′; Hb forward: 5′ - ACCAACCCCAAGCTCA AGAC - 3′ and Hb reverse: 5′ - CTGCCACGCCGTATTTCAAG - 3′; and Mub1-forward: 5′ - CACCGGCAAGGTAACCAG - 3′ and Mub1-reverse: 5′ - GACATAGGTGAGTCCGCAC - 3′, respectively, which were synthesized by Eurofins. Extraction of total RNA was carried out using the RNeasy Plant Mini Kit (QIAGEN) and all of the steps followed the standard protocol of its manufacturer. Concentration of total RNA was measured using NanoDrop 1000 instrument (Thermo Fisher Scientific) and 1.5 μg RNA was loaded onto 1% agarose gel to observe the quality of the extracted RNA. RNase-free DNase I treatment of RNA followed the protocol of Ambion (Thermo Fisher Scientific). Reverse transcription of RNA followed the

manufacturer's protocol for the qScript^{TM} cDNA SuperMix (Quanta Biosciences). The single strand cDNA was used as template in the following PCR. The cDNA synthesized in this step was diluted into a series of cDNA solutions to determine efficiency of the primers. PCR of *ADH3, Hb,* and *Mub1* followed the manufacturer's protocol for the SsoFastTM EvaGreen® Supermix (Bio-Rad Laboratories): 0.5 μl 10 μM forward primer, 0.5 μl 10 μM reverse primer, 1 μl cDNA (diluted eightfold) and 5 μl SsoFast^{TM} EvaGreen® Supermix were mixed and adjusted to 10 μl using nuclease-free water. Samples were transferred to Hard-Shell 96-well plate (Bio-Rad Laboratories) and sealed tightly using a transparent membrane. No template control (NTC) was set up for each biological sample and run in duplicate. Biological replicates corresponded to independent RNA extracts, and three technical replications were run for each biological replicate. The program for the RT PCR reactions was set up as enzyme activation at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 58°C for 30 s. PCR products were viewed by electrophoresis on 2% agarose gel. PCR was performed on a CFX96 Real-Time PCR Detection System and results analyzed with Bio-Rad CFX Manager. Data was expressed as the cycle number required for reaching a threshold fluorescence value (C_q). Data was normalized to the mean C_q of the reference gene, for which variation between samples was <1. The specificity of primer pairs was confirmed by melt curve analysis in comparison with controls without template. PCR efficiency was calculated from a standard curve of C_q versus the logarithm of starting template quantity. Each assay was optimized so that efficiency ranged between 97 and 101%, with a coefficient of determination (R^2) > 0.99.

Determination of *S*-nitrosoglutathione reductase (GSNOR, EC 1.2.1.46) was modified from the method of Wünsche et al. (2011). GSNOR was extracted from embryo with 50 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl and 0.1 mM EDTA. Supernatant was collected after centrifuging for 15 min at 18,000 × *g* and passed through Sephadex G-10 column. Fifty μl of the filtered solution was taken to measure enzymatic activity at 340 nm in final 1 ml of 50 mM Tris-HCl (pH 8.0) containing 0.4 mM GSNO and 0.2 mM NADH ($\varepsilon = 6.22\ mM^{-1}cm^{-1}$). The reaction was initiated by adding GSNO and change of absorbance was recorded for 3 min.

Measurement of Ascorbate and Glutathione

Ascorbate and glutathione were extracted with 6% TCA from tissue powder. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C. Supernatant was used for measurement of reduced and oxidized ascorbate and glutathione. Ascorbate (ASC) and dehydroascorbate (DHA) were determined according to Kampfenkel et al. (1995), and the absorbance was recorded at 525 nm using spectrophotometer. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined according to Zaharieva and Abadía (2003). The method is based on the reaction of DTNB with GSH forming 5-thionitrobenzoic acid (TNB) recorded at 412 nm. The oxidized glutathione (GSSG) was measured after its reduction by glutathione reductase (Sigma).

Measurement of Enzymes Involved in Oxidative Metabolism

The extraction method was modified from Murshed et al. (2008). All extraction steps were performed on ice. Catalase, SOD, alcohol and lactate dehydrogenases were extracted from embryo with 50 mM Tris-HCl buffer, pH 8.0. The enzymes of ascorbate-glutathione cycle were extracted from fine powder of isolated embryo with 50 mM MES-KOH buffer, pH 6.5, containing 40 mM KCl, 2 mM $CaCl_2$, 1 mM ascorbate (for ascorbate peroxidase, added freshly). The ratio of extraction buffer to the scutellum tissue was 1 ml to 20 mg powder of tissue in fresh weight (FW). The samples were vortexed and then centrifuged at 15000 g for 10 min. Supernatant was collected as a crude enzyme solution.

Measurement of SOD (EC 1.15.1.1) was performed according to Kuthan et al. (1986) and Gupta et al. (1993) by inhibition of reduction of cytochrome *c*. SOD was extracted by 50 mM potassium phosphate buffer, pH 7, containing 0.1 mM EDTA and 1% (w/v) polyvinylpolypyrrolidone (PVP). The spectrophotometric buffer contained 0.05 M potassium phosphate (pH 7.8), 0.1 mM EDTA, and 0.05 mM xanthine. Fifty μl of crude SOD solution and 50 μl 5 mg/ml cytochrome *c* on ice were mixed with 0.85 ml of the buffer until absorbance at 550 nm was constant and finally 0.05 ml of xanthine oxidase (0.02 U/ml) was added and mixed. The increase of absorbance at 550 nm was recorded for 2 min. KCN (2 mM) was added to SOD extract to measure activity of MnSOD. The activity of Cu/Zn SOD was calculated as a difference of total SOD and MnSOD. The activity of Fe SOD, which is mainly chloroplastic, was negligible and not considered.

The methods for measuring activities of the ascorbate-glutathione cycle enzymes were modified from Nakano and Asada (1987). The assay medium for ascorbate peroxidase (APX, EC 1.11.1.11) was 50 mM potassium phosphate buffer (pH 7.0) containing 0.25 mM sodium ascorbate and 50 μl sample extract. The reaction was started by adding H_2O_2 (final concentration 0.25 mM) and the reaction rate was determined spectrophotometrically by absorbance change at 290 nm (coefficient of absorbance, $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$). Dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity was measured at 265 nm ($\varepsilon = 14\ mM^{-1}\ cm^{-1}$). The assay buffer contained 50 mM HEPES buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH, and 50 μl of extract. The reaction was initiated by adding freshly prepared DHA (final concentration of 0.8 mM). Monodehydroascorbate reductase (MHAR, EC 1.6.5.4) activity was measured in 50 mM HEPES buffer (pH 7.6) containing 2.5 mM ascorbate, 0.25 mM NADH, and 50 μl of the extract. The assay was initiated by adding 0.4 U cm-3 of ascorbate oxidase and the reaction rate was monitored at 340 nm ($\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). Glutathione reductase (GR, EC 1.8.1.7) activity was measured in 50 mM HEPES buffer (pH 8.0) containing 0.5 mM EDTA, 0.25 mM NADPH, and 50 μl extract. The reaction was started by adding GSSG to final concentration of 1 mM. Catalase (EC 1.11.1.6) activity was measured at 240 nm according to Aebi (1974). In the above reactions, the change of absorbance was recorded for 3 min by Biochrom Ultrospec 4300 spectrophotometer (Amersham, UK).

Alcohol dehydrogenase (ADH; EC 1.1.1.1) was measured according Blandino et al. (1997) with ethanol and NAD^+. Determination of lactate dehydrogenase (LDH; EC 1.1.1.27) activity was performed according to Hoffman et al. (1986) with pyruvate and NADH. Both activities were measured spectrophotometrically at 340 nm.

Statistical Analysis

All the experiments were repeated at least three times. The data in the text, tables, and figures are expressed as means ± SD of three replicates. The differences with $P \leq 0.05$ were considered as statistically significant.

RESULTS

Germination of Barley Seeds

The seeds of Harrington cultivar were non-dormant with the germination rate 96%. The seeds of Sundre cultivar kept the moderate level of dormancy with germination resistance 1.2 days. Practically all seeds were viable, the treatment with gibberellic acid resulted in alleviation of dormancy and germination rate of 96% for both cultivars (not shown). Therefore, the non-germinated seeds in figures represented viable seeds that kept dormancy at the particular time of assay. Radicle protrusion took place between 10 and 15 h post imbibition. **Figure 1** shows the germination of two cultivars as observed at 24 h after imbibition (after radicle protrusion in non-dormant seeds) and at 48 h (when all non-dormant seeds were germinated).

Protein Change, ATP Level and ATP/ADP Ratio

The total protein content in embryo (**Figure 2A**) started to decrease immediately after imbibition. The protein content decreased almost twofold during the first day and further decreased in next 24 h. The seeds of Sundre that remained non-germinated exhibited stable protein content.

The level of ATP in embryo (**Figure 2B**) sharply increased in the first 3 h after imbibition then declined reaching approximately the same level as in dry seeds after 24 h and declined further on the second day after imbibition. The level of ATP in non-germinated Sundre seeds was constant and similar to

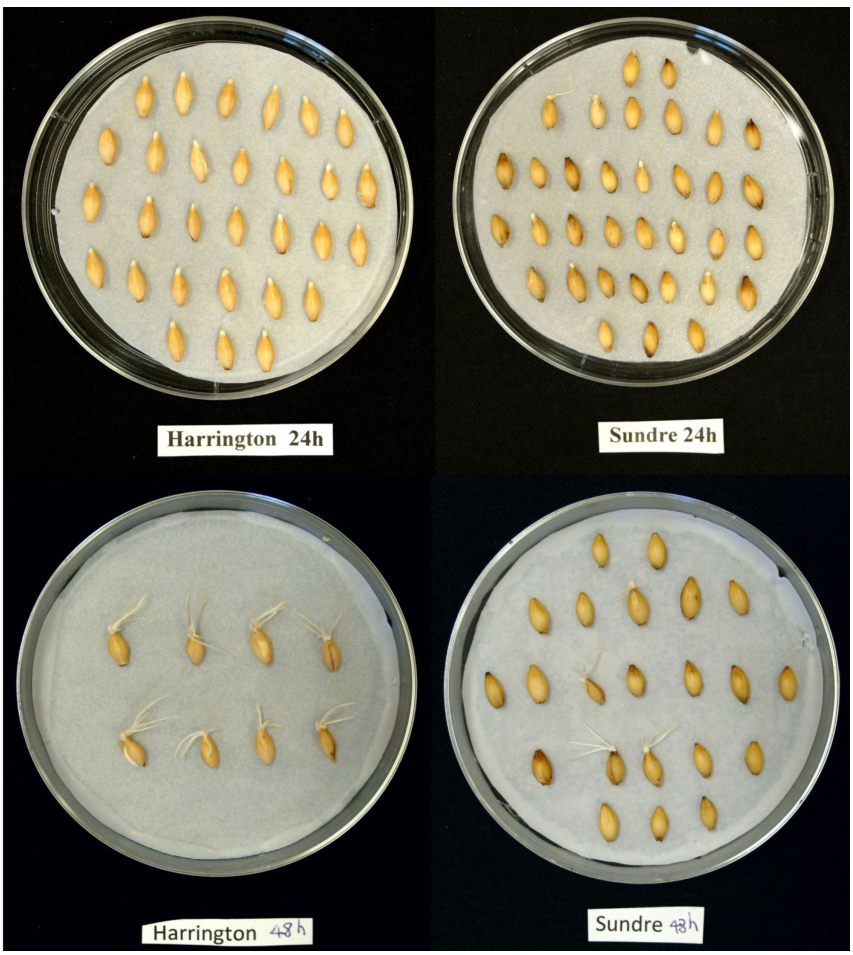

FIGURE 1 | Photos of germinating seeds of Harrington and Sundre cultivars of barley taken at 24 and 48 h post imbibition.

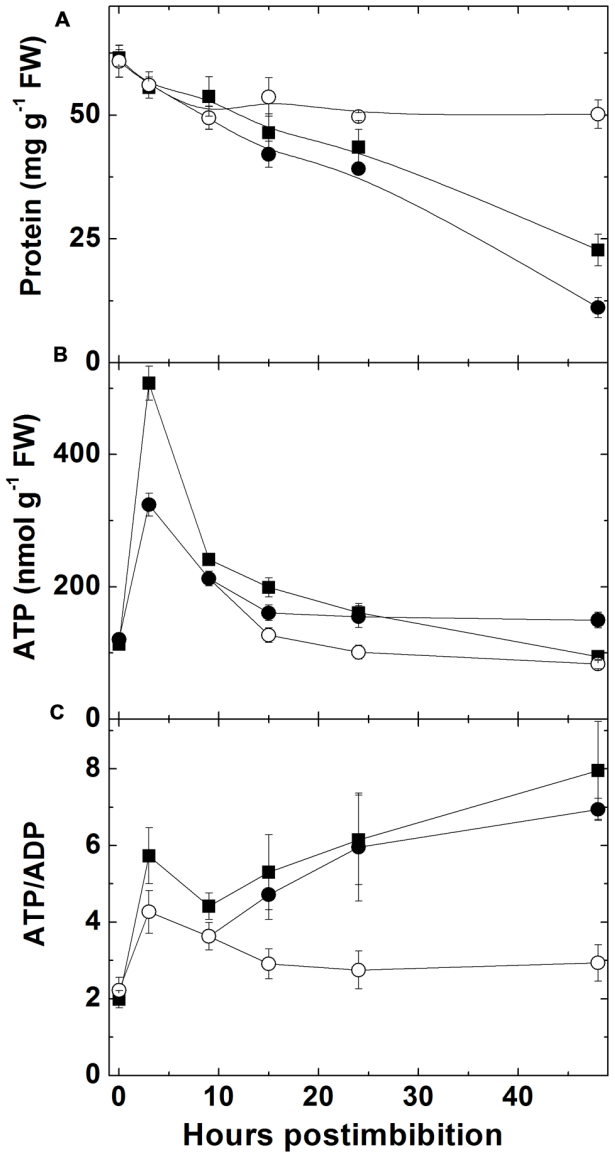

FIGURE 2 | Changes in total protein content (A), ATP (B), and ATP/ADP ratio (C) in embryo of two barley cultivars during germination. Closed squares – Harrington cultivar, closed circles – Sundre cultivar, open circles designate non-germinated seeds of Sundre.

the level in dry seeds. The ratio of ATP and ADP (**Figure 2C**) also increased (from 2 to 6 in Harrington) in first 3 h, then declined to 4 after 10 h post imbibition, and increased again upon radicle protrusion with the same dynamics in both cultivars. The non-germinated seeds of Sundre kept almost the constant ATP/ADP ratio in embryo of approximately 2.5.

NO Metabolism in Embryo of Germinating Seeds

The level of NO, measured with use of hemoglobin, increased (sharply in Harrington) in embryo in the first day starting from imbibition and then the increase became slower (**Figure 3A**). There was no increase in embryo of seeds of Sundre cultivar that remained non-germinated (even some decrease was observed). The increase of nitrosylation of SH-groups of proteins in embryo (**Figure 3B**) was continuously increasing after imbibition (only in the seeds remaining non-germinated this increase was slow). The level of free SH-groups in proteins decreased during germination remaining constant in non-germinated seeds (**Figure 3C**). The lower initial level of NO production in Harrington corresponded to lower rate of nitrosylation in the first hours after germination. The genes encoding the proteins participating in NO metabolism (class 1 phytoglobin and GSNOR) were expressed in germinating seeds (**Figures 4A,B**). While the expression of GSNOR was practically constant during 48 h post imbibition (slightly decreasing in Sundre), the expression of phytoglobin I gene increased in the first hours after imbibition, and started to decline on the second day post imbibition. There was no significant

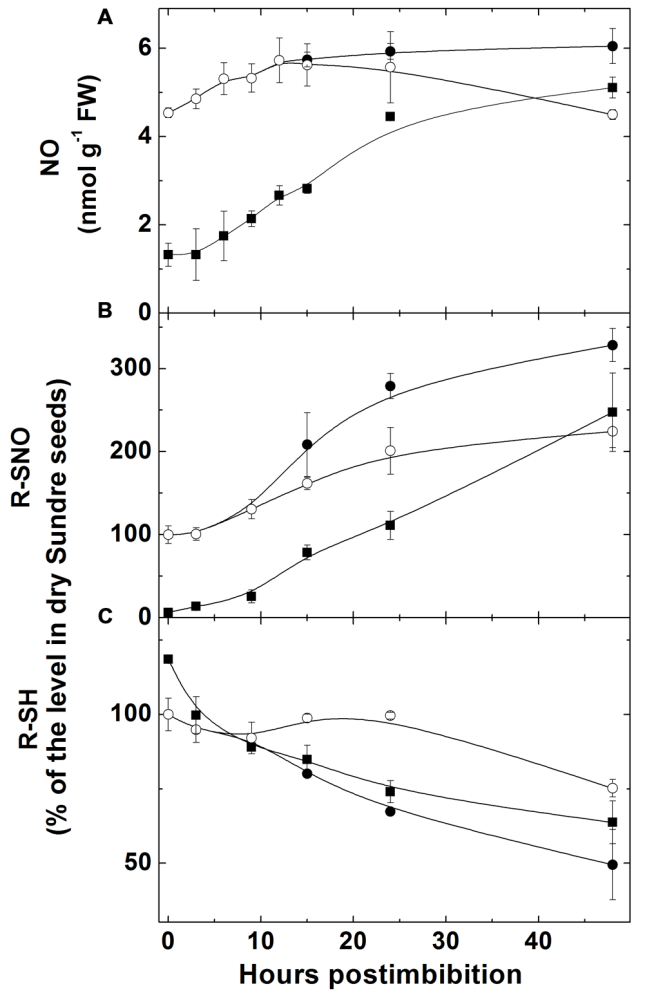

FIGURE 3 | Changes in NO level (A), in the quantity of nitrosylated (−SNO) groups in proteins (B), and the quantity of sulfhydryl groups in proteins (C) in embryo of two barley cultivars during germination. The symbols are the same as in **Figure 2**.

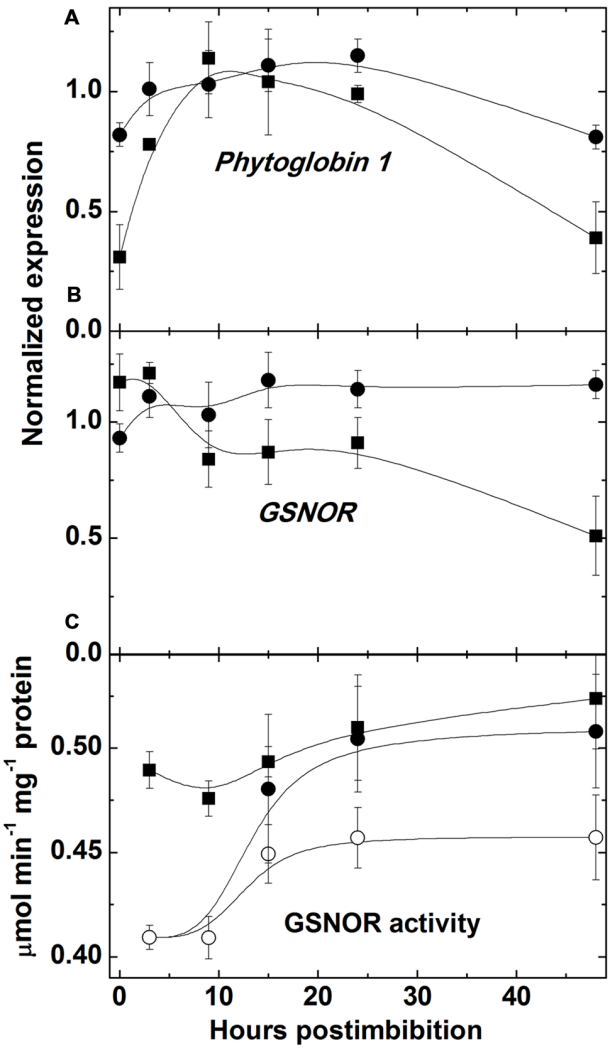

FIGURE 4 | Expression of the genes encoding class 1 phytoglobin (A) and GSNO reductase (B) and GSNO reductase activity (C) in embryo of two barley cultivars during germination. The symbols are the same as in **Figure 2**.

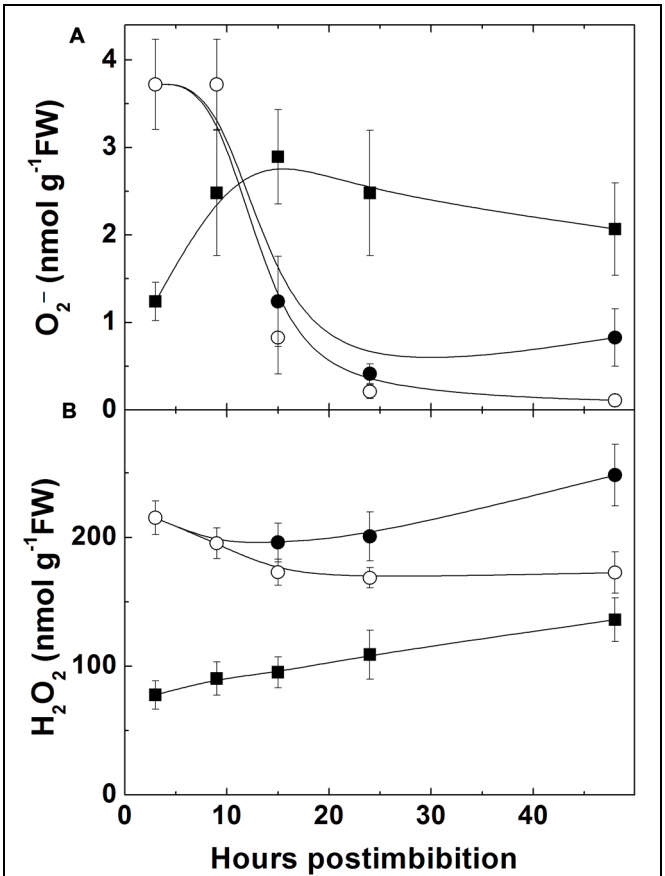

FIGURE 5 | Changes in the level of superoxide anion (A) and of hydrogen peroxide (B) in embryo of two barley cultivars during germination. The symbols are the same as in **Figure 2**.

difference in expression of GSNOR and phytoglobin I genes between germinated and non-germinated seeds of the Sundre cultivar (not shown). The activity of GSNO-reductase increased during germination, more in Sundre than in Harrington, remaining at a lower level in non-germinated seeds (**Figure 4C**).

H_2O_2 and $O_2^{\bullet-}$ Levels

The level of superoxide was high in dry seeds of Sundre and low in Harrington. It decreased in Sundre upon imbibition and increased in Harrington (**Figure 5A**) being significantly higher in embryo after 10 h from imbibition. The content of H_2O_2 (**Figure 5B**) was higher in the Sundre cultivar remaining constant in non-germinated seeds, and having tendency of increase in germinating seeds in both cultivars, with a sharper increase in Harrington.

Ascorbate and Glutathione Levels and Free SH-Groups

The ratio of ASC to DHA (**Figure 6A**) was initially higher in Harrington and then decreased, while the level of reduction of ascorbate pool in Sundre was significantly lower than in Harrington. The total ascorbate pool slightly decreased during the first day post imbibition in both cultivars, in dormant seeds it remained constant (**Figure 6B**). The level of reduction of glutathione decreased in Harrington in the first day, then increased; in Sundre the decrease was observed continuously from the start of imbibition (**Figure 6C**). These changes took place on the background of decrease in the total pool of glutathione (**Figure 6D**).

Activities of Enzymes Involved in Oxidative Metabolism

Catalase activity increased in Harrington in the first hours, then decreased and started to increase on the second day; in Sundre the activity was lower but the increase was continuous (there was no increase in non-germinated seeds; **Figure 7A**). SOD activity drastically increased after a period of low activity and some fluctuations in first 10–15 h (before radicle protrusion), these

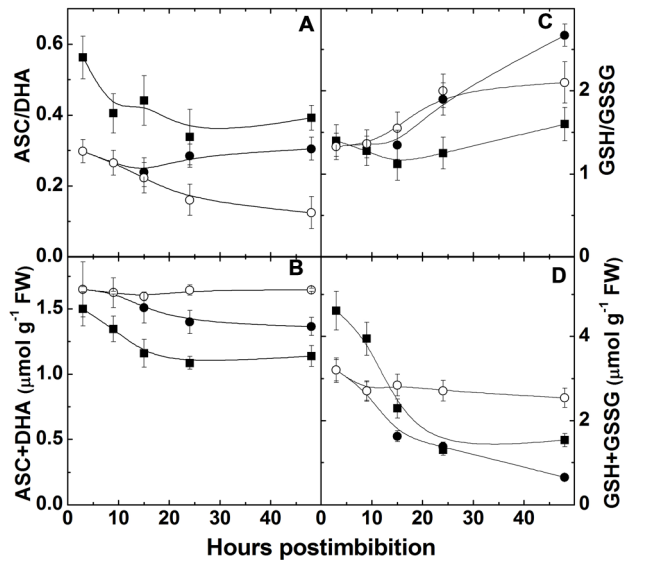

FIGURE 6 | Changes in the ratio ascorbate and dehydroascorbate (A), total ascorbate pool (B), the ratio of reduced and oxidized glutathione (C), and total glutathione pool (D) in embryo of two barley cultivars during germination. The symbols are the same as in **Figure 2**.

changes were similar for Cu/Zn-SOD and Mn-SOD and exhibited the same tendency in both cultivars. It did not show a change in the embryos of the seeds that remained non-germinated (**Figure 7A**).

All four enzymes of the ascorbate-glutathione cycle exhibited low initial activity and increased markedly during the first 48 h after imbibition (**Figure 7B**). Non-germinated seeds of Sundre cultivar kept low level of activity of all four enzymes.

The activities of LDH and ADH were high in the first hours after imbibition and then decreased (**Figure 8**). They were significantly lower in more dormant Sundre cultivar and did not show change in the seeds that kept dormancy.

DISCUSSION

Seed germination is a process that starts from imbibition and results in radicle protrusion initiating seedling development. This process corresponds to the two stages described by Bewley and Black (1994), in the first of which rapid depletion of oxygen takes place and in the second the conditions inside seed are close to anaerobic. After radicle protrusion, the third stage starts in which oxygen concentrations gradually return to aerobic, active mobilization of storage reserves takes place and the seedling begins to develop. In barley seeds, radicle protrusion occurs within the first 24 h after imbibition. In our study the first radicles appeared between 12 and 15 h, and by 24 h all non-dormant seeds developed radicles (**Figure 1**). The separation between the first and second phases is conventional but we assume that by 3–5 h from imbibition most of oxygen is depleted and then the seeds exist in mostly anaerobic conditions until radicle protrusion.

Germination is characterized by the gradual utilization of storage reserves and a buildup of ATP, which is used for biosynthetic processes (**Figure 2**). In the seeds remaining dormant the protein level remains without change and the ATP/ADP ratio is constant being near 2.5. The first peak of ATP level is observed at 3 h post imbibition, which probably corresponds to the peak of aerobic metabolism before oxygen becomes depleted. Higher ATP levels in the non-dormant cultivar might reflect higher respiration capacity which in turn could result in higher production of superoxide in the side reactions of mitochondrial metabolism (Møller, 2001). After reaching its peak ATP level sharply declines, reflecting the development of anaerobic conditions, however, the observed decline in the ATP/ADP ratio is not strikingly pronounced and the ATP/ADP ratio, reaching the value 6 in Harrington at 3 h, declines only to the value slightly higher than 4 at 10 h. This indicates that even under highly hypoxic conditions there exist the mechanisms supporting ATP synthesis. Besides fermentation, NO turnover can be a contributor to ATP synthesis in these conditions (Stoimenova et al., 2007). Higher activities of fermentation enzymes (ADH and LDH) were indeed observed in germinating barley seeds in the first hours post imbibition, and the cultivar Harrington having higher ATP/ADP ratio at the first day is characterized by higher levels of ADH and LDH (**Figure 8**). It is also characterized by a sharper increase in phytoglobin expression (**Figure 4A**), which may also contribute to a buildup of ATP via the phytoglobin-nitric oxide cycle (Stoimenova et al., 2007; Gupta and Igamberdiev, 2011). Phytoglobin gene expression precedes the expression of phytoglobin protein which was detected in barley grains in the earlier study (Duff et al., 1998).

Indeed, the production of NO was shown to increase in barley seeds starting immediately from the onset of imbibition (**Figure 5A**). NO levels were initially higher in Sundre (more dormant cultivar) remaining stable in the seeds that kept dormancy, while in Harrington the increase in NO production was faster. The increase was most pronounced at the initial stages of germination, when the seeds become more anaerobic. This corresponds to the mechanism of reductive pathway of NO formation (via nitrite reduction at high redox level) facilitated by transition to anaerobic conditions (Dordas et al., 2003). The oxidative pathway of NO production is considered to be less active in plants and may be absent in germinating seeds (Igamberdiev et al., 2014). It is known that the development of anaerobic conditions in seeds results in a switch from oxygenic respiration to fermentation. An alternative to fermentation pathway could be NO turnover in the phytoglobin-nitric oxide cycle, in which NO is produced by reduction of nitrite and scavenged with formation of nitrate, the reaction catalyzed by a system involving the class 1 phytoglobin (Igamberdiev and Hill, 2004; Gupta and Igamberdiev, 2011). Both pathways operate during seed germination, and the activities of ADH and LDH (**Figure 8**) and the expression of phytoglobin (**Figure 4A**) exhibit cultivar-specific patterns.

The production of NO leads to nitrosylation of SH groups in proteins, peptides, free cysteine and its derivatives. The redox and energy level of germinating seeds is controlled via the balance

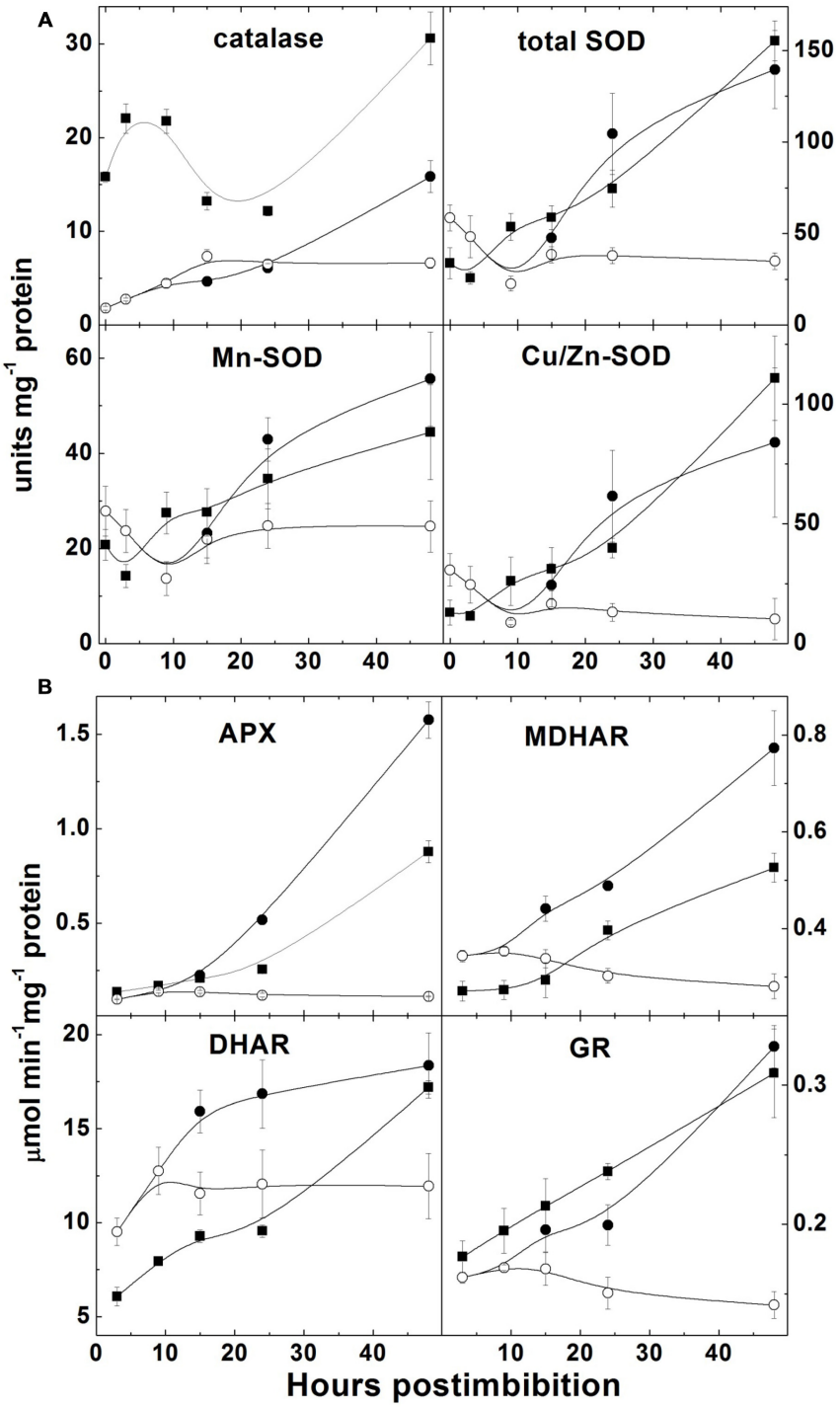

FIGURE 7 | Activities of the enzymes scavenging reactive oxygen species in barley embryos during germination. (A) Activities of catalase and superoxide dismutase (total SOD, Mn-SOD, and Cu/Zn-SOD); **(B)** Activities of the enzymes of ascorbate-glutathione cycle (APX – ascorbate peroxidase, DHAR – dehydroascorbate reductase; MDHAR – monodehydroascorbate reductase; GR – glutathione reductase). Units of catalase are millimoles H_2O_2 min^{-1}; units of SOD are 50% reduction of superoxide formation per minute. The symbols are the same as in **Figure 2**.

of NO producing and scavenging mechanisms (Bykova et al., 2015). The most important scavenging mechanism for NO is its oxygenation to nitrate with participation of class 1 phytoglobin, which is hypoxically induced (Igamberdiev and Hill, 2004). While NO can be scavenged via phytoglobin-mediated mechanism, the most important RNS species, *S*-nitrosoglutathione is scavenged

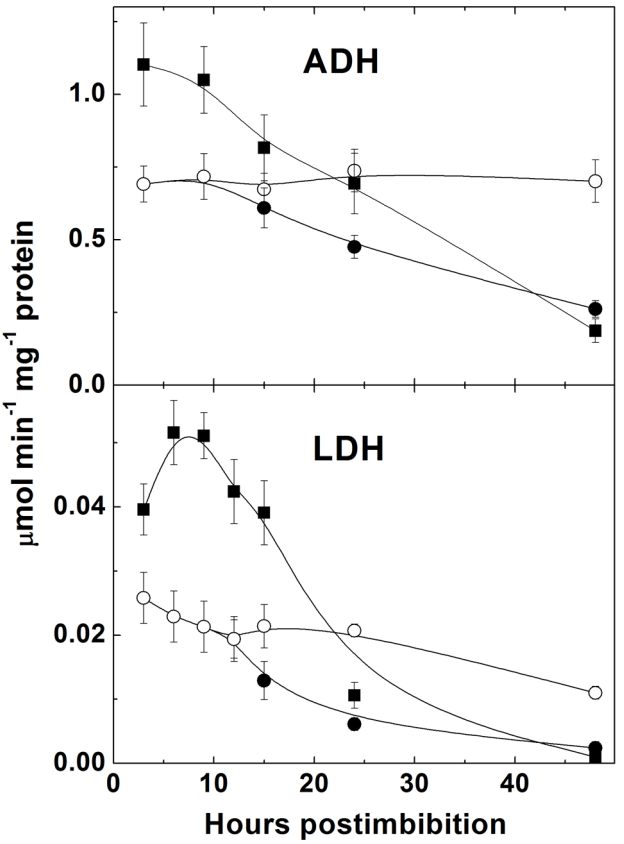

FIGURE 8 | Activities of fermentation enzymes alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) in embryo of two barley cultivars during germination. The symbols are the same as in **Figure 2**.

by GSNOR, which is the class III alcohol dehydrogenase having also the activity of formaldehyde dehydrogenase (Corpas et al., 2013). The activity of this enzyme was high already in dry seeds and it remained higher in the cultivar with high germination rate (Harrington; **Figure 4C**). The transcript expression of this enzyme (**Figure 4B**) was relatively constant from the onset of germination; therefore the observed changes in activity could be attributed to the posttranscriptional regulation. On the contrary, phytoglobin expression started to increase from the beginning of germination, remained high between 3 and 24 h and then declined; the changes were more expressed in the cultivar Harrington (**Figure 4A**). This is in agreement with the reported dependence of phytoglobin expression on the hypoxic conditions (Igamberdiev and Hill, 2004).

While we did not measure the concentration of *S*-nitrosoglutathione in this study, the decline in total glutathione (GSH + GSSG) pool (**Figure 6D**) can be explained, in particular, by its intensive nitrosylation. Other modifications of thiol groups caused by the increase in ROS formation may be responsible for the observed changes. Nitrosylation and other modifications also explain the decrease of free SH-groups in proteins (**Figure 3C**), which is supported by the observed increase of nitrosylated (−SNO) groups (**Figure 3B**), which follows the pattern of NO production with some delay (the increase in nitrosylation becomes more pronounced after 10 h post imbibition). The production of RNS (NO and nitrosylated compounds) during seed germination was suggested to be connected with dormancy alleviation (Bethke et al., 2004) but later it was suggested that inhibition of respiration by NO triggers ROS formation and that results in dormancy alleviation (reviewed in Bykova et al., 2015). We observed that in the seeds remaining dormant the increase in NO did not take place and the nitrosylation process did not actively develop (**Figure 3**).

In the course of germination upon the development of anaerobic conditions we observe oxidation of ascorbate and glutathione pools, while after radicle protrusion (at 15 h post imbibition) the ratios start to increase (**Figures 6A,C**). The glutathione pool and its reduction is the indicator of the overall cellular environment and important in control of morphogenetic processes (Mitrovic et al., 2012). Schafer and Buettner (2001) suggested that the resulting action of the glutathione redox couple triggers metabolic events and determines morphogenesis. GSH stimulates *Arabidopsis* root growth (Sanchez-Fernandez et al., 1997) favoring cell division and proliferation, while the GSSG content is related to differentiation (Schafer and Buettner, 2001; Stasolla et al., 2004). The changes of glutathione pool and its reduction level observed during germination are much more pronounced than of the ascorbate pool (**Figure 6**).

The importance of glutathione pool size and its reduction for determination of cell division, growth, and even apoptosis was mentioned in many works (reviewed in Noctor et al., 2012). The quiescent parts of plants such as root quiescent center, and cells in organs such as seeds maintain a highly oxidized intracellular state, in particular reflected in low content of reduction of the glutathione pool (Kranner et al., 2006), and the increase in the total cellular GSH pool is essential for the cells to progress to cell division (Diaz-Vivancos et al., 2010). Glutathione synthesis is required for pollen germination and pollen tube growth (Zechmann et al., 2011). The direct effect of glutathione has been observed in relation to its potential in meristematic cells; however, total tissue concentrations could give indirect indication of the direction of the morphogenetic process. Our study shows that the total pools of glutathione decreased after imbibition while its reduction increased after radicle protrusion. The redox state of glutathione can be tightly controlled by NO production and scavenging via phytoglobin and GSNOR mechanism. Thus NO can be an important regulator of dormancy release and tissue differentiation during seed germination.

The cross-talk between NO and ROS formation is important even during seed germination. The production of ROS occurs actively upon imbibition even in the period of oxygen depletion (**Figure 5**). Antioxidant defense mechanisms protect seeds during the dormancy stage and prevent their germination, while the break of dormancy is achieved in conditions when ROS production breaks these control mechanisms (Bykova et al., 2011a,b; Bykova and Rampitsch, 2013). Some data relate dormancy alleviation with the accumulation of H_2O_2 (Oracz et al., 2007; Bailly et al., 2008), however, the role of superoxide anion was also considered. It has been demonstrated that germination in *Arabidopsis* is mediated by accumulation of O_2^-

and H_2O_2 in the radicle (Leymarie et al., 2012). In sunflower (*Helianthus annuus* L.) seeds, germination was shown to be associated with a marked increase in hydrogen peroxide and superoxide anion generation in the embryonic axes resulting from an inhibition of catalase and SOD and from activation of NADPH oxidase (Oracz et al., 2009). The role of NO in germination was also claimed (Bethke et al., 2004), and its interaction with ROS results in the balance of both ROS and RNS compounds that provides the conditions for germination.

The increase of superoxide levels in the non-dormant cultivar Harrington and its decrease in more dormant Sundre was observed during the first day, while the levels of H_2O_2 were lower in the seeds of the non-dormant cultivar (**Figure 5**). This might indicate that the main ROS player in alleviating dormancy in barley is indeed superoxide anion. On the other hand, some role of H_2O_2 in promotion of germination follows from the fact that in the seeds remaining non-germinated its level is lower than in the germinating seeds of the Sundre cultivar. The differences between the levels of superoxide and hydrogen peroxide could be related to the activities of corresponding scavenging enzymes (**Figure 7A**). However, before radicle protrusion the activities of SOD and of the enzymes of ascorbate-glutathione cycle were low and they started to increase drastically mostly on the second day of germination. The higher activity of catalase in Harrington may explain the lower H_2O_2 level at the onset of germination. Cakmak et al. (1993) also reported that during imbibition of wheat seeds the increase in activities of H_2O_2 scavenging enzymes is much more pronounced than the increase in SOD. In our study the increase in SOD (both the mitochondrial Mn-SOD and cytosolic Cu/Zn-SOD) was observed mainly on the second day of germination, i.e., after radical protrusion and switch to aerobic metabolism. The role of ascorbate-glutathione cycle is important not only for scavenging of H_2O_2 but also for establishing redox potentials of ascorbate and glutathione, which are the best indicators of the overall cellular environment and important in control of morphogenetic processes (Mitrovic et al., 2012; Talukdar, 2012).

CONCLUSION

The production of NO and ROS during germination and their cross-talk are the important events that take place during germination of barley seeds. The class 1 phytoglobin, GSNOR and interaction with ROS play a role in scavenging and turnover of NO and nitrosylated compounds during germination. Although the observed differences between the two cultivars may be not necessarily be attributed to the dormancy levels, it becomes evident that the turnover of NO contributes to the maintenance of redox and energy balance of germinating seeds and may be an important player in alleviation of dormancy.

AUTHOR CONTRIBUTIONS

ZM performed all experiments, participated in discussion of results, contributed to writing the manuscript. FM planned and supervised the experiments on gene expression, analyzed results, contributed to writing the manuscript. NB planned and supervised the experiments on measurement of enzymes and metabolites, analyzed results, contributed to writing the manuscript. AI supervised the design, execution and interpretation of the experiments, prepared the manuscript.

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Effects of NO_2 and Ozone on Pollen Allergenicity

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This mini-review summarizes the available data of the air pollutants NO_2 and ozone on allergenic pollen from different plant species, focusing on potentially allergenic components of the pollen, such as allergen content, protein release, IgE-binding, or protein modification. Various *in vivo* and *in vitro* studies on allergenic pollen are shown and discussed.

Keywords: pollen, allergens, NO_2, ozone, air pollution

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INTRODUCTION

Allergic diseases are increasing in Europe. The potential reasons discussed for this trend include climate change and anthropogenic air pollution (Kramer et al., 2000; Ring et al., 2001). It has been shown that ozone and NO_2, which are two important air pollutants, can have an adverse effect on human health, e.g., the lung function, such as the initiation of lung inflammation by ozone (Kagawa, 1985; Uysal and Schapira, 2003). These air pollutants not only affect human health but also impact plants and their pollen. Ground level ozone, which is a secondary pollutant, is the result of a photochemical reaction of volatile organic compounds and nitrogen oxides (NO_x), which are mainly produced by combustion processes during energy production, industrial processing or car traffic. Ozone again can also interact with NO_x. In rural areas, NO_2 concentrations of up to 20 ppb can be measured, whereas in urban traffic regions, values up to 90 ppb are detectable. In case of ozone, ozone levels along high traffic roads are often low, since ozone is depleted due to the high NO-concentration. At rural sites ozone concentrations often are higher, even so less precursors components are available, since polluted air is transported to the country sites and can react to ozone while the transport. The information threshold for ozone is 1-h average of 90 ppb, but on sunny days alert threshold level of 120 ppb 1-h average can be reached (www.eea.europa.eu).

Air pollution can impact pollen morphology, the pollen cell wall, the pollen protein content or protein release from the pollen as well as the pollen protein itself. In addition, the pollen coat, which is a complex mixture of pigments, waxes, lipids, aromatics and proteins (Edlund et al., 2004), might be impaired due to air pollution. Regarding pollen allergenicity, not only allergenic proteins do play a role, but pollen-derived lipids, which are also called pollen-derived lipid mediators (PALMs), also interact with the immune system and can modify the allergenic reaction (Traidl-Hoffmann et al., 2003; Bashir et al., 2013).

THE IMPACT OF OZONE ON POLLEN ALLERGENICITY

Concerning the effect of traffic-/industrial-related air pollution on allergenic pollen, most studies have been interested in the allergens, including the allergen content, the possibility of allergen/protein release from the pollen or possible modifications to the allergens. Interestingly,

approximately one-fourth of known plant allergens are pathogen-related proteins (PR-proteins), which are induced by different biotic and abiotic stresses, including ground-level ozone (Sandermann et al., 1998; Hoffmann-Sommergruber, 2002). One example is Bet v 1, the major allergen from birch (*Betula pendula*), which is a PR-10 protein. Beck et al. (2013) showed that the Bet v 1 allergen content is positively correlated with increasing ozone levels. This *in vivo* study also indicated significantly larger wheal and flare sizes in patients pricked with pollen extracts from high ozone stands, which was consistent with the higher Bet v 1 content in the pollen (Beck et al., 2013). Another PR protein known as an allergen is the thaumatin-like protein Cup a 3 from *Cupressus arizonica*, which was shown to increase under polluted air conditions (Cortegano et al., 2004; Suárez-Cervera et al., 2008). A further study on another tree species, *Pinus radita,* showed greater allergenicity in terms of prick test and specific IgE binding in connection with higher ozone levels in unpolluted areas (García-Gallardo et al., 2013). The sensitivity to ozone seems to be both species- and concentration-dependent. In an *in vitro* study with ozone concentrations of half, equal and four times the standard limit according to the European Union Directive 2008/50/EC on ambient air quality, the pollen of *Acer negundo*, *Platanus* ssp. and *Quercus robur* showed differences in the total soluble protein (TSP) content. Whereas *Q. robur* and *Platanus* ssp. exhibited significantly reduced protein content under all treatments, *A. negundo* only showed a decrease when it was fumigated with the highest ozone concentration (Ribeiro et al., 2013). Concerning the specific IgE reactivity to *Q. robur* and *A. negundo* pollen extracts, the majority of the tested sera showed increased or unchanged IgE activity compared to the control, but for *Platanus* ssp., the untreated samples showed the highest IgE binding (Ribeiro et al., 2014). Increased allergen contents due to elevated ozone have also been shown for other plant species, such as *Lollium perenne* and *Secale cereale* (Masuch et al., 1997; Eckl-Dorna et al., 2010). A study on grass pollen (*Phleum pratense*) fumigated *in vitro* with 100 ppb ozone for 4 hours resulted in the acidification of several allergens (Phl p 1 b, 4, 5, and 6) as well as decreased IgE recognition of the allergens Phl p 1, 2, 6, and 13 in immunoblots, as explained by the mechanical loss of allergens from altered pollen grains (Rogerieux et al., 2007; **Table 1**). The exposure of *Phleum pratense* pollen to increasing ozone-concentrations from 100 ppb up to 5 ppm resulted in a significant increase in the naturally released pollen cytoplasmic granules (PCG), which are also known to contain allergens, and in more damage to the pollen grain. This mechanism of allergen release might explain the increase in thunderstorm asthma (Motta et al., 2006). Two other studies on *in vivo* and *in vitro* fumigated ragweed pollen did not find any differences in the allergen content of the major allergen Amb a 1 (Pasqualini et al., 2011; Kanter et al., 2013), but differences in the pollen cell wall and increased NADPH oxidase activity could be detected. NADPH oxidase activity was already shown to influence allergenic reactions due to the release of reactive oxygen species (Bacsi et al., 2005). In addition, cell wall modifications might affect immune reaction: in ozone-fumigated ragweed pollen, reduced levels of wax compounds have been detected, and high ozone levels resulted in an altered lipid composition of birch pollen, which led to a modulated immune response (Beck et al., 2013; Kanter et al., 2013).

NO_2 IS IMPACTING THE POLLEN ALLERGENICITY

Several studies have been performed examining the influence of NO_2 on pollen allergenicity. Three *in vitro* studies exposing

TABLE 1 | Studies of the effect of NO_2 and/or ozone on the allergenic potential of different pollen species.

Species	Pollutant	Concentration	Exposure time	Allergen	IgE binding	Reference
Acer negundo	O_3	30–235 ppb	6 h		↑	Ribeiro et al., 2014
	NO_2	150–300 ppb	6 h		↑	Sousa et al., 2012
Ambrosia artemisiifolia	O_3	80 ppb	Growing season	Amb a 1=		Kanter et al., 2013
	O_3	100 ppb	7 day	Amb a 1=		Pasqualini et al., 2011
	NO_2	80 ppb	Growing season	Amb a 1 ↑	↑	Zhao et al., 2015
	NO_2	High traffic roads			↑	Ghiani et al., 2012
Betula ssp.	O_3	Outside stands		Bet v 1 ↑		Beck et al., 2013
Betula pendula	NO_2	34/67 ppb	6/48 h		↑	Cuinica et al., 2014
Carpinus betulus	NO_2	34/67 ppb	6/48 h		↑	Cuinica et al., 2014
Lolium perenne	O_3	65 ppb outside stands	2 weeks –	Phl p 5 ↑ Phl p 5 ↑		Masuch et al., 1997
Phleum pratense	O_3 NO_2 O_3/NO_2	100 ppb 2000 ppb 100 ppb/2000 ppb	4 h		↓ ↓ ↓	Rogerieux et al., 2007
Pinus radita	O_3	Outside stands			↑	García-Gallardo et al., 2013
Secale cereale	O_3	80 ppb	107 days	Phl p 1 ↑ Phl p 5 ↑ Phl p 6 ↑ Profilin ↑		Eckl-Dorna et al., 2010

↑ = *increased;* ↓ = *reduced.*

grass pollen (*P. pratense*) to artificially high NO_2-concentrations from 500 ppb up to 5000 ppm resulted in a dose-dependent increase in PCGs and a dose-dependent increase in pollen grain damage (Motta et al., 2006) as well as a reduction of IgE binding to Phl p 2, 5b, and 6 (Rogerieux et al., 2007). In addition, a direct correlation of NO_2 uptake by the pollen with a higher T_H2 response of human cells could be demonstrated (Chassard et al., 2015), which indicated that NO_2 can interact with the pollen grains and thus leads to degradation of pollen structure and changes in protein content. Other studies on ragweed (*Ambrosia artemisiifolia*) showed higher allergen levels and elevated IgE binding under elevated NO_2 concentrations, where the higher IgE recognition was mainly due to the major allergen Amb a 1 (Ghiani et al., 2012; Zhao et al., 2015). Zhao et al. (2015) also showed IgE binding to a new allergen in ragweed with homology to Hev b 9 from the rubber tree, induced by elevated NO_2 (Zhao et al., 2015). However, contrary results were found for several tree pollen species fumigated with NO_2 concentrations between 34 and 300 ppb. *Betula pendula* and *Carpinus betulus* showed decreased pollen viability when exposed to NO_2 and had a lower TSP content. *Ostrya carpinifolia* also showed a lower TSP compared with the control. This study also demonstrated increased pollen allergenicity for *C. betulus*, *O. carpinifolia*, and *B. pendula* when exposed for a short time to relatively small NO_2 concentrations (Cuinica et al., 2014). *A. negundo* also showed similarly elevated IgE binding but slightly increased TSP, whereas lower TSP was detected for *Ricinus communis* (Bist et al., 2004; Sousa et al., 2012).

Air pollution due to NO_2 can result in post-translational modification such as S-nitrosylation or the nitration of pollen proteins. Zhao et al. (2015) showed that the fumigation of ragweed plants with elevated NO_2 concentrations throughout a growing season resulted in increased overall S-nitrosylation, and LC-MS/MS analysis of the S-nitrosylated proteins indicated the major ragweed allergen Amb a 1 as a possible candidate for S-nitrosylation (Zhao et al., 2015). Another important aspect is the nitration of allergens in pollen, which can be caused by the presence of ozone and NO_2. Studies on aerosolized proteins showed an ozone dependent increase of nitration due to NO_2 (Shiraiwa et al., 2012). Franze et al. (2005) showed that the major allergen from birch Bet v 1 is effectively nitrated in the presence of NO_2 and ozone but that the nitration degree was substantially lower when the proteins were exposed to NO_2 alone, thus indicating that reactive species formed upon the interaction of ozone and NO_2 play a major role in the nitration. This nitration affects the allergenic potential of the birch pollen. The nitration of Bet v1 results in stronger proliferation of Bet v 1-specific T cell lines, and IgE binding to nitrated Bet v 1 is higher than IgE binding to Bet v 1 (Gruijthuijsen et al., 2006; Karle et al., 2012). An oligomerization of Bet v 1 due to nitration was also observed, which resulted in lower sensitivity to endolysomal degradation (Ackaert et al., 2014). This posttranslational modification of allergens provides a rationale for the increase in allergic diseases in air polluted regions.

Summarizing the different studies, little is yet known about the molecular mechanisms of the effects of ozone and NO_2 on pollen, and more research is needed on that point. However, the existing research already clearly indicates dose-dependent and species-specific impacts of these air pollutants, which in most cases result in heightened allergenicity.

AUTHOR CONTRIBUTIONS

UF and DE contributed equally to the manuscript concerning writing and conception.

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NADPH Oxidase-Dependent Superoxide Production in Plant Reproductive Tissues

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In the life cycle of a flowering plant, the male gametophyte (pollen grain) produced in the anther reaches the stigmatic surface and initiates the pollen–pistil interaction, an important step in plant reproduction, which ultimately leads to the delivery of two sperm cells to the female gametophyte (embryo sac) inside the ovule. The pollen tube undergoes a strictly apical expansion characterized by a high growth rate, whose targeting should be tightly regulated. A continuous exchange of signals therefore takes place between the haploid pollen and diploid tissue of the pistil until fertilization. In compatible interactions, theses processes result in double fertilization to form a zygote (2n) and the triploid endosperm. Among the large number of signaling mechanisms involved, the redox network appears to be particularly important. Respiratory burst oxidase homologs (Rbohs) are superoxide-producing enzymes involved in a broad range of processes in plant physiology. In this study, we review the latest findings on understanding Rboh activity in sexual plant reproduction, with a particular focus on the male gametophyte from the anther development stages to the crowning point of fertilization. Rboh isoforms have been identified in both the male and female gametophyte and have proven to be tightly regulated. Their role at crucial points such as proper growth of pollen tube, self-incompatibility response and eventual fertilization is discussed.

Keywords: NADPH oxidase, NOX, pollen, pistil, pollen–pistil interaction, Rboh, sexual plant reproduction

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SUPEROXIDE GENERATION IN PLANTS

The superoxide radical ($O_2^{\bullet -}$), a short-lived reactive oxygen specie (ROS) characterized by moderate reactivity, is able to trigger a cascade of reactions (enzymatic, metal-catalyzed, and even direct reactions) in order to produce others ROS species. $O_2^{\bullet -}$ is produced in chloroplasts, mitochondria, the endoplasmic reticulum, and peroxisomes due to their normal metabolism (Sharma et al., 2012).

Apart from the production of free radicals that occurs as a result of the side reactions of metabolism and electron leakage, the plant oxidative burst was first described as the physiologically-controlled and rapid ROS generation during the early responses to pathogen infections, similarly to what occurs in animal phagocytic cells. This process has been described as involving the activity of NADPH oxidase enzymes, called Rbohs, in plants (Lamb and Dixon, 1997). Although the Rboh family seems to be the major source of the generated ROS as described above, other systems, including cell wall-bound peroxidase (POXs)

(Bolwell et al., 2002), oxalate oxidase (Hu et al., 2003), amine oxidase (Angelini and Federico, 1989), and quinone reductase (Schopfer et al., 2008), have been proposed. With regard to plant POXs, $O_2^{\bullet-}$ generation occurs via the H_2O_2−dependent POX cycle as well as via the H_2O_2−independent oxygenation cycle (Kimura et al., 2014). An apoplastic peroxidase-catalyzed oxidative burst following biotic stress has been described in different species such as *Arabidopsis thaliana* or *Phaseolus vulgaris* (Bolwell et al., 2002; O'Brien et al., 2012), although NADPH oxidase and other sources including mitochondria also contribute to ROS generation (O'Brien et al., 2012). On the other hand, the oxidative response induced by hypo-osmolarity in cell cultures has been shown to originate from NADPH oxidase activity in tobacco, whereas, a cell wall-POX capable of producing superoxide, has been identified in *Arabidopsis* (Rouet et al., 2006). In a different study dealing with intact and excised sunflower roots, distinct sources of superoxide were detected: extracellular POX appeared in both samples, while NADPH oxidase was present in intact roots only (Garrido et al., 2012). The studies mentioned above analyze the relative contributions of peroxidases and NADPH oxidases to ROS production by using inhibitors which affect enzymes differently (Bindschedler et al., 2006; Daudi et al., 2012). Thus, the effect of cytochrome inhibitors potassium cyanide and sodium azide, was compared with the impact caused by diphenyleneiodonium (DPI), which is capabable of inhibiting Rboh activity by affecting dimerization and mobility (Hao et al., 2014). Likewise, the ability of Cd^{2+} ions to inhibit NADPH oxidase was used to distinguish between NADPH oxidase -produced $O_2^{\bullet-}$ and the superoxide derived from mitochondria (Heyno et al., 2008).

NADPH OXIDASE-DEPENDENT OXIDATIVE BURST: A PLANT OVERVIEW

NADPH oxidase enzymes belong to a family of transmembrane proteins able to transport electrons across a membrane -usually the plasma membrane- from a cytosolic electron donor to oxygen -the extracellular acceptor- thus catalyzing the generation of $O_2^{\bullet-}$ (Lambeth, 2004). However, the lifespan of superoxide molecules, which are rapidly dismutated to H_2O_2 either spontaneously or through the action of apoplastic superoxide dismutases (SOD), is extremely short (Bowler et al., 1994). NADPH oxidase (NOX) activity was first described in mammal phagocytic cells and consists of two plasma membrane proteins, gp91phox and p22phox (phox for phagocyte oxidase). This protein complex is regulated by its interaction with other cytosolic proteins (p47phox, p67phox, p40phox, and Rac2) producing the active form (Cross and Segal, 2004; Lambeth, 2004). Although these mammalian protein complexes are the most studied NOXs, these enzymes have also been characterized in other animals, fungi, and plants. NADPH oxidase enzymes share a basic structure consisting of six transmembrane domains, two heme-binding sites and a long cytoplasmic C-terminal which includes FAD and NADPH binding sites (Bedard et al., 2007).

In plants, the *Rboh* gene was first isolated in *Oryza sativa* as a homolog of the *gp91phox* mammal gene, which was later described in tomato, *Arabidopsis*, potato, and tobacco (Groom et al., 1996; Keller et al., 1998; Torres et al., 1998; Amicucci et al., 1999; Simon-Plas et al., 2002; Yoshioka et al., 2003). The *Arabidopsis* genome encodes at least 10 Rboh homologues, which usually show different gene expression patterns both in different plant organs and at different developmental stages, suggesting that these isoforms have no overlapping function (Torres et al., 1998; Dangl and Jones, 2001). Nevertheless, *RbohD* appears to be highly expressed in the whole plant and probably acts as a housekeeping gene (Hao et al., 2014). Rbohs were initially described as plasma membrane proteins (Sagi and Fluhr, 2001; Takeda et al., 2008) associated with specific plasma membrane microdomains, and with sterols being regarded as crucial for a proper localization (Liu et al., 2009; Posé et al., 2009). In fact, their distribution, which is not uniform, is characterized by discrete dynamic spots with a highly heterogeneous diffusion coefficient (Hao et al., 2014). In the same study, the effective amount of the isoform analyzed (RbohD) was shown to be regulated by both endocytosis and transport to the vacuole for degradation. Rboh accumulation has actually been detected not only in the plasma membrane but is also associated with internal endomembranes (Heyno et al., 2008; Drerup et al., 2013). According to these findings, Rboh-dependent ROS were also detected in vesicles in response to salt stress and during ABA-induced stomatal closure (Leshem et al., 2007, 2010). Although these locations could initially be considered to be surprising, the mammalian NOX2 enzyme has also been found in endosomes as well as at the plasma membrane; NOX4 has been reported to accumulate in intracellular membranes, the endoplasmic reticulum and even in nuclear compartments (Ushio-Fukai, 2006).

Nowadays, the physiologically controlled production of $O_2^{\bullet-}$ by Rboh proteins is known to be implicated in signaling functions related to a variety of processes involved in biotic interactions (plant responses to pathogens and establishment of functional symbiotic nodules) and also related to abiotic stress responses and adaptations (heat, drought, cold, high light intensity, salinity, and wounding); Rbohs also play critical roles in a broad range of developmental functions such as cell growth, both diffuse, and polarized (Marino et al., 2012). Furthermore, Rboh-derived $O_2^{\bullet-}$ has been shown to mediate cell-to-cell communication over long distances in plants (Miller et al., 2009).

THE ACTIVITY OF PLANT NADPH OXIDASE IS MULTIREGULATED

Unlike in animals, NADPH oxidase activity in plants does not require specific interactions with protein partner homologous to the mammal phagocyte protein complex described above. As described below, Rboh proteins possess an N-terminal region involved in specific regulatory mechanisms; this leads signal transduction pathways -namely calcium, protein phosphorylation and lipid signaling- to connect to ROS production (Suzuki et al., 2011).

As with NOX5- and DUOX-type NOX proteins in animals, Rboh proteins display an N-terminal extension with EF-hand motifs (Bedard et al., 2007). Their presence is crucial in

Rboh activity, as EF-hands motifs are involved in Ca^{2+}-based regulation (Keller et al., 1998) and dimer formation, which has been proposed as the active conformation (Oda et al., 2010). The first crystal structure of the Rboh N-terminal region shows four EF hands, two of which are responsible for dimer stabilization through domain swapping (EF1 of one molecule interacts with EF2 of another molecule and *vice versa*), the remaining two hands being EF-type motifs not predicted from the sequence (Oda et al., 2010). Ca^{2+} binding to EF-hand motifs is essential to trigger oxidative burst, and $O_2^{\bullet-}$ production stimulated in this way, in turn, activates Ca^{2+} channels that lead the cation influx into the cell, thus acting as positive feedback (Foreman et al., 2003; Takeda et al., 2008). It is sufficient for Ca^{2+} to bind to only one of the EF hands in order to produce a conformational change in the EF-hand region, which could act as a molecular switch (Ogasawara et al., 2008; Oda et al., 2010). In mammalian NOX5, the binding of Ca^{2+} to EF-hand motifs induces a conformational change that enables the EF-hands to interact with the C-terminus, which is thought to stimulate the enzyme activity (Bánfi et al., 2004). This direct intramolecular interaction between the N- and C-termini also occurs in plant NADPH oxidases, although, unlike in animals, it has been shown to be Ca^{2+} independent and to require the whole N-terminal region (Oda et al., 2010).

In addition to direct Ca^{2+} binding, calcium-regulated protein families, like some calcium-dependent protein kinases (CDPKs) and calcineurin B-like protein-interacting protein kinases (CIPKs), can stimulate Rboh activity via phosphorylation. Two CDPKs from potato were able to induce $O_2^{\bullet-}$ production through phosphorylation of two serine residues at the N-terminal extension of StRbohB (Kobayashi et al., 2007; Asai et al., 2013). Using this signaling pathway, Ca^{2+} could act as an oxidative burst inducer through the binding to EF-hands of both Rbohs and CDPKs, which, in turn, phosphorylate, and activate Rboh, ultimately leading to ROS production. In *Arabidopsis*, direct Ca^{2+} binding as well as Ca^{2+}-induced phosphorylation by CIPK26 cause a synergistic activation of AtRbohF (Drerup et al., 2013; Kimura et al., 2013). This synergistic effect has also been observed in AtRbohD (Ogasawara et al., 2008), although negative regulation of this isoform by means of a calmodulin-dependent MAPK after wound stress has also been described, suggesting the presence of a feedback pathway to control ROS homeostasis (Takahashi et al., 2011).

Apart from the Ca^{2+}-based regulatory mechanism, calcium-independent direct phosphorylation has also been shown. During plant immunity, the plasma-membrane-associated kinase BIK1 (Botrytis-induced kinase1) directly phosphorylates specific residues of RbohD sites in a calcium-independent manner to enhance ROS production (Kadota et al., 2014; Li et al., 2014). Previously, phosphorylation-induced activation had been proposed as a pre-requisite for Ca^{2+}-mediated activation, placing phosphorylation at the beginning of the plant Rboh-derived ROS signaling pathway (Kimura et al., 2012). Kadota et al. (2015) have proposed a model integrating Ca^{2+}-based regulation and Ca^{2+}-independent phosphorylation, in which it is suggested that the latter 'primes' RbohD activation by increasing sensitivity to Ca^{2+}-based regulation, through Ca^{2+} binding and Ca^{2+}-based phosphorylation. The existence of several different kinases acting in sequential or parallel pathways during defense responses has actually been proposed after multiple phosphorylated residues in RbohD were observed (Benschop et al., 2007). The activating effect of Ca^{2+} and phosphorylation on Rboh activity described above could be exerted by increasing the diffusion coefficient, the dimerization state and the clustering in membrane microdomains (Hao et al., 2014).

Small GTPases such as OsRac1 are thought to activate NADPH oxidase activity in order to trigger oxidative burst during plant-pathogen interaction (Ono et al., 2001). Also, Rboh-derived ROS formation in the growing root hair has been shown to depend on the Rop GTPase (Jones et al., 2007), with the receptor-like kinase (RLK) FERONIA (FER) acting as the upstream regulator of the pathway (Duan et al., 2010). A connection with the regulatory mechanism described above is suggested in the model proposed by Wong et al. (2007), where Ca^{2+}-dependent phosphorylation leads to a conformational change in Rbohs that facilitates Rac binding. This effect could subsequently be suppressed by means of ROS-induced Ca^{2+} accumulation, which would act as negative feedback of NAPH oxidase activity. The Rac-Rboh interaction takes place in the coiled-coil region created by EF-hand swapping, with dimers being suggested to be the functional units for the binding (Oda et al., 2010).

Zhang and co-authors have shown that phosphatidic acid (PA) produced by phospholipase Dα1 (PLDα1) interacts with the N-terminal part of AtRbohD during the ABA response, thus activating ROS production with the downstream involvement of nitric oxide (NO) (Zhang et al., 2009). NO had previously been reported to be linked to ROS at several levels (Delledonne et al., 2001; Delledonne, 2005) and to act as a negative regulator of NADPH oxidase through S-nitrosylation of a conserved Cys in the C-terminal part, which has been suggested to be a conserved mechanism to regulate immune responses in both plants and animals (Yun et al., 2011).

Other less documented interactions include the induction of NADPH oxidase-dependent superoxide production in *Arabidopsis* leaves by extracellular ATP, with cytosolic Ca^{2+} also probably being involved in this signaling network (Song et al., 2006). Finally, RbohD isoform activity has recently been linked to vesicle trafficking. Thus, clathrin-dependent pathways as well as the presence of membrane microdomains affect protein endocytosis, which changes the amount and mobility of the protein at the plasma membrane (Hao et al., 2014).

NADPH OXIDASE ACTIVITY DURING DEVELOPMENT OF THE FEMALE GAMETOPHYTE

In angiosperms, the female gametophyte or megagametophyte, is the embryo sac originating from a haploid megaspore. During gametogenesis, mitochondria have been established as the primary source of mainly superoxide and hydrogen peroxide (Martin et al., 2014a). Although ROS have been shown to be not just involved in but also tightly regulated in megagametogenesis (Martin et al., 2013, 2014b), little information on ROS/superoxide during female gametophyte development is available. These

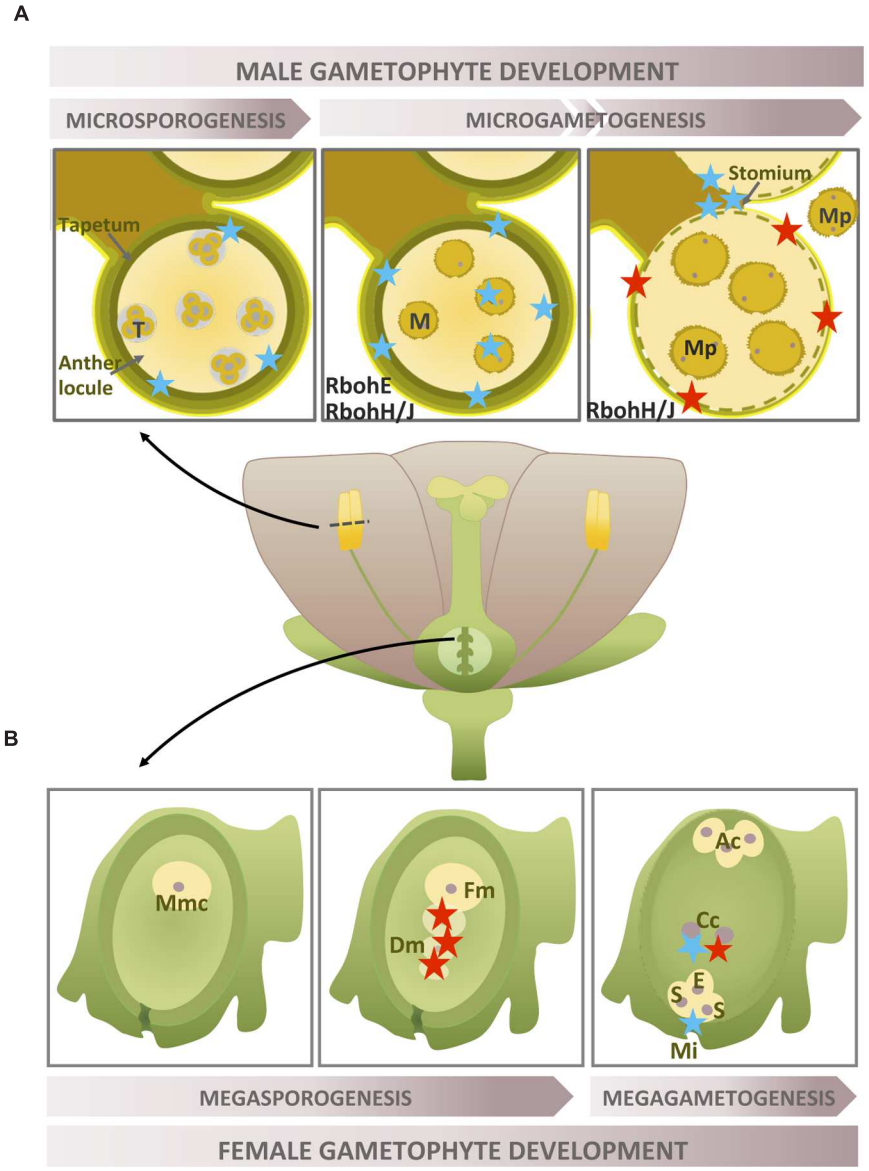

FIGURE 1 | Rboh-dependent superoxide production during gametophyte development. Location of $O_2^{\bullet-}$ during the development of gametophytes. **(A)** Two selected stages of late anther development (tetrads and microspores) and anthers at the dehiscence stage. **(B)** Three selected female gametophyte development stages, including the pre-meiotic phase, functional megaspore formation and the fully developed embryo sac stage. The localization of H_2O_2 accumulation is also shown when necessary (for more detailed information, see text above). Recognized sources of superoxide are marked in black. Blue star: $O_2^{\bullet-}$; red star: H_2O_2; T, tetrads; M, microspore; Mp, mature pollen; Mmc, megaspore mother cell; Fm, functional megaspore; Dm, degenerating megaspore; Ac, antipodal cell; Cc, central cell; S, synergid; E, egg cell; Mi, micropyle.

studies analyze female gametophytic mutants impaired in MnSOD activity which show infertility caused by various defects ranging from development arrest to aberrant egg apparatus. In WT ovules, ROS (H_2O_2) were detected early on in the process, when megaspore cell death takes place (**Figure 1B**). At a later stage of development, the mature female gametophyte showed both mitochondrial superoxide and peroxide accumulation in the central cell, whereas cytosolic superoxide was only detected outside the embryo sac at the micropylar portion (**Figure 1B**). Alternatively, as the mutant ovules showed abnormally high levels of ROS, including cytosolic superoxide, the authors suggest that other sources of ROS, such as NADPH oxidase, might also be involved. Transcriptome data concerning ovule development have demonstrated that *RbohD* shows the highest expression, whereas other Rboh isoforms display quite low expression levels (Wynn et al., 2011). To our knowledge, no female gametophyte-specific Rbohs have been described to date.

NADPH OXIDASE ACTIVITY DURING DEVELOPMENT OF THE MALE GAMETOPHYTE

Superoxide/ROS production during pollen ontogeny has been little studied. Superoxide was detected during rice anther development (Hu et al., 2011) and showed a stage-dependent type of production; it peaked when the formation of young microspores took place and exhibited low levels during the remaining stages and increased slightly when pollen achieved maturation (**Figure 1A**). The subcellular location of $O_2^{\bullet-}$ was determined in the tapetal cells and microspores and may be associated with the initiation of tapetal programmed cell death (PCD). The tapetum is the innermost sporohytic layer in the anther and undergoes cell degeneration to support pollen development after meiosis (Wu and Cheun, 2000). In olive flowers, anthers did not show large amounts of superoxide until the dehiscence stage, when it was localized in the stomium. At this developmental stage, massive production of superoxide together with other ROS (mainly H_2O_2) could also be involved in PCD mechanisms, affecting the endothecium and the surrounding connective tissues as well as the stomium (Zafra et al., 2010). Few data are available on the origin of anther superoxide apart from the involvement of RbohE in the tapetal PCD in *Arabidopsis* anthers (Xie et al., 2014). Transcriptome data concerning microgametogenesis analyzed from the haploid microspore to the mature functional pollen in *Arabidopsis* showed that *RbohH/J* are highly expressed in both the immature and mature tricellular pollen (Honys and Twell, 2004).

PUTATIVE ROLES OF NADPH OXIDASES DURING POLLEN-STIGMA INTERACTION AND POLLEN TUBE GROWTH THROUGH THE FEMALE TISSUES

Pollination

The constitutive accumulation of H_2O_2 at the stigmatic papillae when receptivity peaks has been reported in a large number of angiosperms (**Figure 2A**), and the presence of potential crosstalk with pollen-generated NO has been suggested (McInnis et al., 2006; Zafra et al., 2010). However, the enzymatic source of this H_2O_2 remains unknown. Recently, ROS accumulation upon pollination has been shown to occur in a Rboh-dependent manner in the apoplast/cell wall of the pollen tube touching the stigmatic papillae, since *Arabidopsis rbohH rbohJ* double mutants lacking NADPH oxidase activity are deficient in this feature (Kaya et al., 2015). These authors have proposed that Rboh-generated ROS could assist pollen tube elongation by facilitating the generation of a flexible cell wall tip.

On the other hand, production of cytosolic superoxide as well as peroxide, described following pollination in the *Arabidopsis* embryo sac, is restricted to the synergid cells and is maintained until the pollen tube arrives (Martin et al., 2013, 2014b). The authors believe that the presence of ROS depends on pollination, suggesting that the female gametophyte is able to sense a signal preceding pollen tube contact, although no information on the superoxide source involved is provided. Furthermore, analysis of transcriptomic data during pollen–pistil interactions once again showed that RbohD is the highest expressed isoform in the pistil (Boavida et al., 2011).

Onset of Pollen Germination

Just before the pollen tube emerges, the cytoplasm of the hydrated mature pollen undergoes major changes such as cytoskeleton reorganization, vesicle accumulation near the aperture where pollen tube emerges as well as local thinning of the intine. Apart from these structural alterations, there is also evidence of changes in the presence and localization of ROS (**Figure 2A**). Higher concentrations of ROS are produced in both the cytoplasm and cell wall of the pollen grains at the very beginning of the germination process (Smirnova et al., 2014). These extracellular ROS, specifically H_2O_2, are known to be involved in pollen grain activation (Speranza et al., 2012). Among the ROS involved in pollen germination, $O_2^{\bullet-}$ was detected following pollen rehydration and later at the germination aperture. This occurrence of $O_2^{\bullet-}$ is prevented by the NADPH oxidase inhibitor DPI, which also reduces pollen germination in tobacco and kiwi fruit (Potocky et al., 2012; Speranza et al., 2012).

Allergenic pollen grains have been reported to contain NADPH oxidase activity that differed in intensity and localization according to the plant families studied. Thus, the activity was described at the pollen surface and in the cytoplasm, in subpollen particles released from pollen and at the inner pollen surface, and, in all cases, was mostly concentrated in insoluble fractions (Wang et al., 2009). The presence of this enzyme in numerous allergenic species led the authors to suggest that there is a possible link between NADPH oxidase activity/ROS and allergy, which has been further tested by other authors (Bacsi et al., 2005; Boldogh et al., 2005). However, the findings available in the literature on this issue are contradictory (Shalaby et al., 2013).

After the pollen tube emerges, NADPH oxidase-produced $O_2^{\bullet-}$ appears only in the growing tip and has been proposed as a key element in cell polar expansion (Potocky et al., 2012). This hypothesis has been discussed by others authors, who suggest that superoxide influences pollen germination only indirectly, as it can be spontaneously transformed into H_2O_2, which subsequently, together with OH, regulates tobacco pollen germination by affecting the mechanical properties of the intine (Smirnova et al., 2014). It is important to note that some discrepancies have been reported regarding the role played by NADPH oxidase activity in certain species, such as cucumber, in which DPI and catalase were found to slightly promote pollen germination instead (Sirova et al., 2011).

Pollen Tube Growth

Tip growth consists of elongation exclusively at the apex through the polarized exocytosis of the membrane's newly synthesized components to the apical elongation domain. Furthermore, tip-growing cells, such as pollen tubes, or root hairs, are sensitive to pistil and soil environments, thus making signaling an important

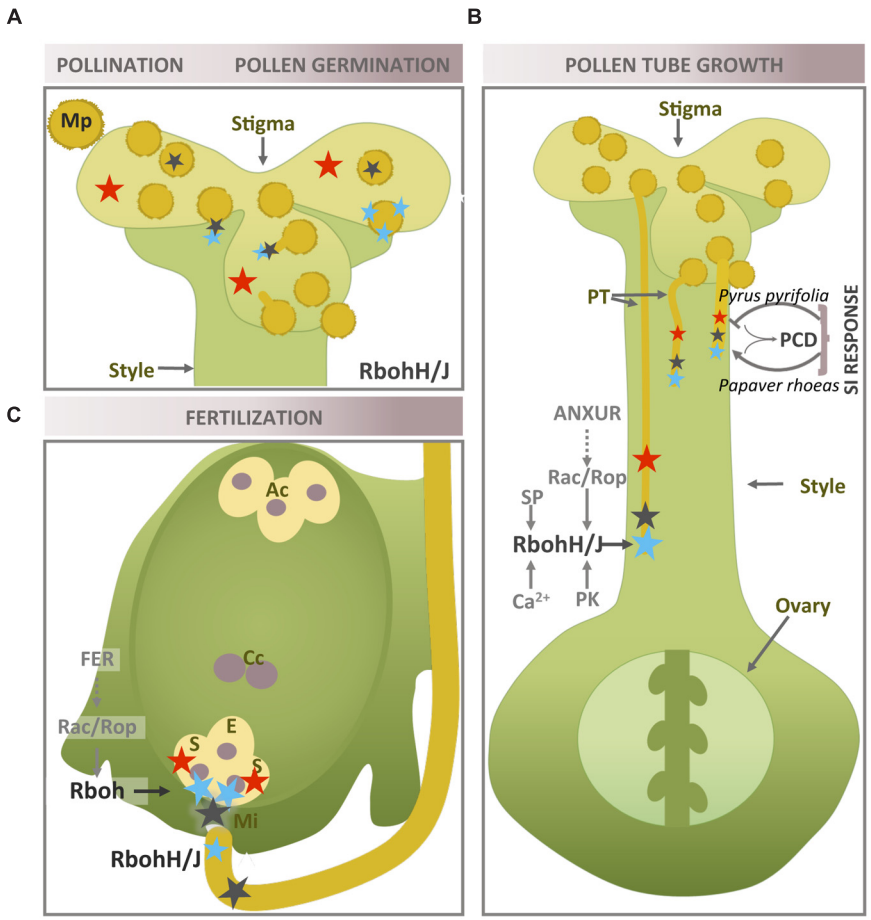

FIGURE 2 | Rboh-dependent superoxide production in plant reproductive tissues during pollen–pistil interaction. Location of $O_2^{\bullet-}$ in reproductive tissues during pollination and pollen germination **(A)**, pollen tube growth **(B)** and fertilization **(C)**. The location of H_2O_2 and NO is also shown when necessary (more information in text above). Recognized superoxide sources and regulatory elements are marked in black. Blue star: $O_2^{\bullet-}$; red star: H_2O_2; black triangle: NO; PT, pollen tube; Ac, antipodal cells; Cc, central cell; S, synergid; E, egg cell; PK, protein kinase; SP, signaling phospholipid; Mi, micropyle.

feature in these kinds of cells (Feijo et al., 2004). Several molecules, including ROS and Ca^{2+}, have been reported to be involved in apical growth (Coelho et al., 2008). It is important to consider the role of other ions, such as potassium, the effect of pH, lipid signaling and small GTPases in the context of cytoskeletal reorganization and polar vesicle trafficking (Feijo et al., 2004; Cole and Fowler, 2006; Lee and Yang, 2008) as well as NO and cGMP (Prado and Feijó, 2009).

NADPH oxidase activity is involved in apical growth in roots, pollen tubes and in the polarized zygotic growth of the model alga *Fucus serratus* (Foreman et al., 2003; Potocky et al., 2007; Coelho et al., 2008). In root hair cells, NADPH oxidase activity is located at the growing tip during elongation, causing superoxide accumulation in the cell wall which facilitates rapid elongation (Dunand and Penel, 2007) and disappearing when growth stops (Takeda et al., 2008). Rboh mediates cell elongation through the production of local ROS waves at the root hair tip (Monshausen et al., 2007) which, in turn, could activate Ca^{2+} channels (Foreman et al., 2003).

In *Arabidopsis thaliana*, two genes *RbohH* and *RbohJ* are specifically expressed in the pollen grain and the growing pollen tube (Sagi and Fluhr, 2006; Kaya et al., 2014). Initial approaches to the study of pollen-specific Rbohs linked the presence of superoxide at the pollen tube apex to the activity of these enzymes, as this process was affected by transfection with specific *Rboh*-antisense oligodeoxynucleotides (ODNs) and by DPI. These treatments also inhibited pollen tube growth, leading to the conclusion that superoxide production by NADPH oxidase activity is essential for apical elongation. Moreover, as previously observed for other isoforms, pollen NADPH oxidase activity is stimulated by calcium (Potocky et al., 2007; Speranza et al., 2012). However, a recent study has suggested that the decrease in DPI-mediated growth could be due to the inhibition of other flavoenzymes in addition to Rbohs (Lassig et al., 2014). In fact, mitochondria were also proposed as the source of these ROS in *Lilium formosanum* (Cardenas et al., 2006). In this pollen, mitochondrial NAD(P)H dehydrogenase activity correlates with the oscillation of the growth rate. These authors observed the

highest ROS production at the subapical region, where most mitochondria are located, rather than at the tip. Moreover, in the cucumber pollen tube, both these localizations occurred at different times: although ROS and NO were detected at the tip at the beginning of tube growth process, their presence then extended to the whole tube (Sirova et al., 2011). In this line of argument, superoxide production was detected in kiwi fruit pollen with no clear localization at the tip (Speranza et al., 2012). To reconcile these contrasting views, other authors have suggested the presence of two ROS sources in growing pollen tubes (**Figure 2B**): NADPH oxidase at the very tip and mitochondria in the subapical region (Liu et al., 2009). Nevertheless, a recent study has shown that the pollen-specific Rbohs, RbohH and RbohJ, from *A. thaliana*, show overlapping localization up to a point: they both appeared in the subapical region very close to the tip, although RbohJ is the only isoform present in the pollen shank (Lassig et al., 2014).

With regard to subcellular localization, initial analyses carried out using gel blotting and determination of cell fraction activity (Potocky et al., 2012) followed by further experiments with fusion proteins (Boisson-Dernier et al., 2013; Kaya et al., 2014) have shown that pollen Rboh isoforms are situated at the plasma membrane of the pollen tube. Targeting depends on endosomal recycling, with RbohH and RbohJ showing differences in internalization kinetics (Lassig et al., 2014). With respect to the pollen plasma membrane, it has been suggested that Rboh proteins are partially included in specific lipid microdomains in *Picea meyeri* (Liu et al., 2009). In addition, lipid microdomain polarization at the tip during pollen tube growth was shown to be necessary for NADPH oxidase activity, the establishment of a calcium gradient and subsequent apical expansion. Curiously, although they would be expected to be situated in the plasma membrane only, RbohH and RbohJ are actually also localized at the cytoplasm (Lassig et al., 2014).

Controlling Pollen Tube Growth Rate and Cell Wall Integrity

In growing pollen tubes, ROS produced by NADPH oxidase activity have been shown to regulate the balance between cell wall extensibility and strength, this structure's two main cytomechanic properties (Carol and Dolan, 2006). ROS production could be involved in two counteracting processes: loosening of the cell wall and cross-linking of cell wall components (Smirnova et al., 2014). In fact, the critical role played by ROS in the cytomechanic characteristics of the cell wall has also been demonstrated in anther tissues. In these tissues, the chloroplast redox system, comprised of proteins containing the cystathionine β-synthase domain CBSX, thioredoxins and peroxiredoxins, is able to connect plant nutritional information and pollen release by controlling the extracellular level of hydrogen peroxide during anther dehiscence (Ok et al., 2012; Jung et al., 2013).

A recent study investigates the *rbohH rbohJ* double mutant, which shows unstable growth as compared to the low fluctuation rates found in WT lines (Lassig et al., 2014). According to this study, two growth patterns were established in this double mutant: (a) periods with short bursts of growth followed by growth cessation and (b) periods with elevated average growth rates, which eventually culminated in pollen tube collapse. The authors of the study established a pivotal role for RbohH and RbohJ, which involves modulating growth rate oscillations in order to facilitate coordination with exocytosis. This original proposal is based on the fact that the double mutant undergoes thinning of the apical cell wall due to the lack of building material, which ultimately forces the pollen tubes to stop growth. This is followed by a thickening of the cell wall owing to apical cell wall deposition or even a collapse due to the excessive increase in exocytosis. NADPH oxidase-produced ROS appears to act as a speed regulator presumably by rigidifying the cell apex. Apart from mediating in cell elongation and shaping the pollen tubes, it has been suggested that pollen apoplastic ROS mediate cell wall loosening and facilitate pollen tube growth through female tissues (Kaya et al., 2015).

The importance of pollen Rbohs in maintaining cell wall integrity during tube growth is also highlighted in a study (Boisson-Dernier et al., 2013) in which it is reported that over-expression of *RbohH* and *RbohJ* causes over-activated exocytosis and accumulation of secreted membrane. This results in aberrant tube morphology, thus suggesting that RbohH/J activity should be tightly regulated.

REGULATING NADPH OXIDASE ACTIVITY IN POLLEN

Many of the aforementioned regulatory mechanisms present in vegetative Rbohs caused by physiological or stress conditions have also been described for pollen Rbohs (**Table 1**).

The second messenger Ca^{2+} and several Ca^{2+}-associated proteins are well-known key elements involved in pollen tube growth, as a tip-focused Ca^{2+} gradient as well as oscillations in intracellular concentrations are required (Cole and Fowler, 2006; Cardenas et al., 2008). As mentioned above, plant Rbohs contain two Ca^{2+}-binding EF-hand motifs in the cytoplasmic portion. In an initial approach, extracellular calcium was used to increase/activate pollen NADPH oxidase activity *in vitro* and *in vivo*, which was observed to have a dose-dependent effect (Potocky et al., 2012). Kaya et al. (2014) went further and, by means of the transitory expression of *RbohH* and *RbohJ* and the induction of Ca^{2+} influx, showed that both proteins effectively displayed Ca^{2+}-activated NADPH oxidase activity. Once again, it was demonstrated that the presence of EF-hands motifs is essential for this activity, as the mutation in two critical positions for Ca^{2+} quelation in the binding loop leads to impaired Ca^{2+}-induced NADPH oxidase activity. The authors also suggest that, as with RbohD, the conformational change in the second EF-hand region is required. In turn, pollen Rbohs affect Ca^{2+} dynamics in the growing tubes; in the *Arabidopsis rbohH rbohJ* double mutant, the tip-focused Ca^{2+} gradient was destabilized and reduced rather than eliminated. Furthermore, an increase in external Ca^{2+} partially rescued the phenotype (Boisson-Dernier et al., 2013). In this study, the authors suggest that pollen Rbohs are not required to generate a Ca^{2+} gradient, which, however, is adjusted and stabilized to support regular growth,

TABLE 1 | Rboh multiregulation in vegetative tissues vs. the pollen tube.

	Vegetative tissues	Pollen tube	Modulation
Ca^{2+}	Direct binding to EF hands motifs Indirectly: Ca^{2+}-induced phosphorylation; binding to Rop-GTPase		Synergistic activation
Phosphorylation	CPK5 CIPK26 MAPK cascades	Unidentified candidates (pollen tube CPK proposed)	Synergistic activation
Small GTPases	Rac1 Rop2	Rop1 Rac5	Activation Targeting?
Low abundance of signaling phospholipids	PA produced by PLDα1	PA (synergism with Ca2+) PIP2	Activation
NO	S-nitrosylation	Putative, not described	Negative

as suggested previously with respect to RbohC in *Arabidopsis* root (Monshausen et al., 2007). Moreover, Lassig et al. (2014) report that, in this double mutant, the steady tip-focused gradient is replaced by intracellular Ca^{2+} bursts preceded by short growth peaks followed by growth cessation. Accordingly, they propose that NADPH oxidase activity is an indirect modulator of intracellular Ca^{2+} dynamics, although which factor operates upstream seems to be unclear. Positive feedback appears to occur during pollen tube growth, with NADPH oxidase activity depending on Ca^{2+} and the ROS produced then maintaining the tip-focused Ca^{2+} gradient. In fact, as in the case of *Arabidopsis* root hairs, ROS-activation of Ca^{2+} permeable channels in the plasma membrane of pollen tubes has been detected (Wu et al., 2010).

Synergistic activation of NADPH oxidase activity by Ca^{2+} and phosphorylation has also been reported (Kaya et al., 2014), although RbohH/J activation through phosphorylation lacks identifiable candidates. Although several CDPKs involved in pollen tube growth could be proposed, current models are based on experimental data from Rboh isoforms that differ from those present in pollen (Wudick and Feijo, 2014). Similarly, the low-abundant signaling phospholipids PA and phosphatidylinositol 4,5 biphosphate (PIP_2) were found to promote NADPH oxidase activity both *in vitro* and *in vivo,* (probably in a synergistic way with calcium), and PA is also likely to play a role downstream of NADPH oxidase activity. In a similar set of experiments with tobacco pollen, small GTPases from the Rop family were shown to act as pollen Rbohs regulators *in vivo*. Moreover, protein targeting to the pollen tube tip could be affected by Rop GTPases (Potocky et al., 2012), although the relationship between Rop and Rboh proteins needs further investigation (Kaya et al., 2014).

Moreover, the pollen ANXUR RLKs, located at the tip-growing tube, have been placed upstream of RbohH and RbohJ and acts as a regulatory element in the pollen tube growth (Boisson-Dernier et al., 2013). As discussed above, Rbohs from root hairs are known to be activated by RLKs through Rop signaling (Duan et al., 2010), although this pathway has not yet been observed in pollen tubes (Boisson-Dernier et al., 2013). Recently, Wudick and Feijo (2014) have developed a clarifying model of ROS signaling in the pollen tube showing the positive feedback discussed above. In this model, small GTPases together with RLKs are considered to be promoters of ROS-production. They describe an initial phosphorylation step, which facilitates Ca^{2+} binding to pollen Rbohs and subsequent ROS production, which, in turn, could activate Ca^{2+} channels. The increase in calcium could, in turn, activate some CDPKs, with a consequent intensification of Rboh phosphorylation and ROS production. Downstream of RbohH/J, a receptor-like cytoplasmic kinase (RLCK), named MARIS (MRI) expressed preferentially in pollen tubes and root hairs, has recently been added to this signaling cascade which seems to ultimately control cell wall integrity during pollen tube tip growth (Boisson-Dernier et al., 2015).

SELF-INCOMPATIBILITY (SI) RESPONSE TO NADPH OXIDASE ACTIVITY

The self-incompatibility (SI) response consists of a collection of molecular and cellular mechanisms capable of preventing self-fertilization by suppressing pollen germination and tube growth. Redox signaling by reactive species (ROS/NO) has been shown to be clearly involved in these processes. SI is based on stigma and pollen recognition, with three main SI models driven by different genes having been proposed, all three of which use a multiallelic S-locus system (Takayama and Isogai, 2005). The combination of different haplotypes enables discrimination between compatible and incompatible interaction- rejection of incompatible pollen occurs through different mechanisms, including PCD, which is ultimately triggered in incompatible pollen tubes. ROS, together with NO, are key actors in the course of PCD in plants (Wang et al., 2013), and their involvement in the SI response is highlighted in **Figure 2B**. However, as SI systems and S-determinants have yet to be determined in many plant families (Dresselhaus and Franklin-Tong, 2013), these signaling mechanisms require further study (Serrano et al., 2015).

Pear (*Pyrus pyrifolia* L.) shows an S-RNase-based gametophytic SI mechanism, consisting of a specific inhibition of self-pollen germination and tube growth by the style S-RNase. As already discussed above, tip-localized ROS are necessary for pollen tube growth; nevertheless, the pollen tubes of incompatible pear pollen showed a disruption in NADPH oxidase- and mitochondria-mediated ROS production (Wang

et al., 2010). The authors show how this SI-mediated ROS disruption elicits a decrease in Ca^{2+} as well as actin cytoskeleton depolymerization and nuclear DNA degradation - the latter two processes being considered key markers of PCD (Wang et al., 2013) – and how DPI and a ROS scavenger are used to support the involvement of Rboh and ROS. Interestingly, an extracellular apoplastic calmodulin (CaM) from the transmitting tissues of the pistil managed to rescue self-pollen tube growth *in vivo* and *in vitro*, possibly through the induction by Ca^{2+} of current and subsequent generation of tip-localized superoxide/ROS and through stabilization of actin filaments. In addition, CaM-dependent stabilization of actin filaments could occur through cross-talk between cytosolic free calcium and ROS (Jiang et al., 2014). However, direct evidence of Rboh involvement has not been obtained.

Papaver rhoeas displays a different SI mechanism in which the S-locus includes pollen and pistil S-determinants, whose interaction in an incompatible combination results in the rapid inhibition of pollen tube tip growth involving PCD. This mechanism initially involves a Ca^{2+}-dependent network, which leads to a relatively rapid and temporary increase in the levels of ROS and NO (Wilkins et al., 2011). Both reactive species act upstream of SI markers such as DEVD/caspase-3-like activity and actin cytoskeleton reorganization, suggesting that ROS and NO together activate PCD in the SI response. The sharp rise in ROS is diffusely localized in the tube shank and in indeterminate spots, whose origin is controversial, as the authors propose sources different, at least in part, from the NADPH oxidase. In olive trees (*Olea europaea* L.), PCD has been involved in the rejection of incompatible pollen by arresting its growth in the style. During *in vitro* pollen germination, the increase in tip-localized superoxide in the course of self-incompatible experiments was accompanied by an increase in the gene expression of some Rboh isoforms (Serrano et al., 2012). The authors suggest that superoxide as well as NO are key signaling molecules in the interaction between the incompatible pollen and the pistil, which can trigger PCD programs (**Figure 2B**).

With regard to the other SI model for *Brassicaceae*, no clear involvement of ROS/NO has yet been described. Nevertheless, in this model, the different molecular mechanisms involved in the redox signaling role played by thiol-based redox protein thioredoxins have been highlighted and outlined (Cabrillac et al., 2001; Ivanov and Gaude, 2009).

FERTILIZATION AND SUPEROXIDE PRODUCTION

The ultimate goal of pollen tube growth is to ensure that the female gametophyte delivers the sperm nuclei. Once again, the characterization of the two *Arabidopsis Rboh* genes specifically expressed in pollen, using the respective single and double mutant, shows the important role played by these genes in the fertilization process. Self-fertilization rates in the single *rbohH* and *rbohJ* mutants were comparable to wild-type rates (Kaya et al., 2014). However, the double mutant, which was partially sterile, was rarely found in the progeny (Boisson-Dernier et al., 2013) and had shorter siliques and fewer seeds than WT (Boisson-Dernier et al., 2013; Kaya et al., 2014; Lassig et al., 2014). This is explained by the mutant pollen's inability to reach the female gametophyte *in vivo,* while most of the pollen tubes were actually found to break up during *in vitro* germination. In other words, the disruption of RbohH and RbohJ prevented the fertilization of the female gametophyte. Boisson-Dernier et al. (2013) have suggested that the functional redundancy of RbohH and RbohJ is partial, with RbohH being able to compensate for the loss of RbohJ, although this substitution was only partial in the opposite direction. Moreover, the authors observed tube rupture during *in vitro* germination in the *rbohH* single mutant, although this phenotype did not significantly reduce seed production. NO has also been shown to be involved in ovule micropyle addressing (**Figure 2C**) (Prado et al., 2008). RbohH and RbohJ could be putatively involved in ovule targeting, as both proteins carry the conserved Cys residue, which is effectively nitrosylated in RbohD (Yun et al., 2011). However, this post-translational modification has still not been described in pollen.

As ROS are detected in the synergid cells upon the arrival of the pollen tube (Martin et al., 2014a), NADPH oxidase activity could be involved in plant fertilization through another mechanism. Once pollen tube has entered the embryo sac via the micropyle, the female gametophyte induces its rupture, and the sperm cells are released and made available for fertilization. A recent study has shown that female gametophyte NADPH oxidase-dependent ROS are necessary for this ovule task to succeed (Duan et al., 2014). ROS generated at the entrance of the embryo sac (filiform apparatus/synergid cell region) reach a maximum level, coinciding with maximum ovule receptivity; the ROS cause both this tube rupture, which is Ca^{2+}-dependent *in vivo* and *in vitro,* and sperm release. Thus, the application of ROS to *Arabidopsis* pollen tubes leads to a sharp increase in Ca^{2+} in the distal cytoplasm, immediately followed by pollen tube rupture, suggesting the presence of Ca^{2+} -signaling events downstream in the pollen tube. As suggested in relation to root hairs, the FERONIA receptor-like kinase (FER) acts upstream of Rop. FER is broadly expressed except in pollen, where, as previously mentioned, the pollen-specific ANXUR homologs are upstream of RbohH and RbohJ, (**Figures 2B,C**) (Boisson-Dernier et al., 2013).

After fertilization, the exclusion of ROS from the fertilized embryo sac is required for the embryo to develop properly, although no biological significance has been proposed in relation to the remaining superoxide at the micropylar end of the integuments (Martin et al., 2013, 2014b).

CONCLUSION

In this study, we review evidence on the critical involvement of ROS generated by plant NADPH oxidases in a broad range of processes related to sexual plant reproduction. From anther development to pollen germination and pollen tube elongation through polarized growth until fertilization, several plant Rboh isozymes have been identified as specifically expressed in the

male gametophyte, where they play important reproductive roles under tightly regulated conditions. Although the identified forms of Rboh appear to show at least partially redundant functions, some questions remain in relation to the regulation of RbohH/J in the pollen tube. For example, the positive feedback mediated by Ca^{2+} appears to occur in pollen, where, as occurs with roots hairs, RbohH/J-produced ROS may activate as yet unidentified plasma membrane Ca^{2+} permeable channels. This positive feedback is probably a conserved mechanism at the apex of tip-growing cells, which could be involved in maintaining polarity. Such an important regulatory mechanism as phosphorylation in Rboh isoforms continues to constitutes a gap in the RbohH/J model, as phosphorylation agents (Ca^{2+}-dependent and non-Ca^{2+}-dependent) have not yet been identified. Consistent with the findings on vegetative tissues, small GTPases from the Rop/RAC family affect Rboh activity in pollen tubes, although further studies are necessary in order to investigate this interaction and its putative effect on RbohH/J localization. In root hairs, FER activates RbohC-dependent ROS production through ROP2 signaling, whereas, in pollen tubes, ROP involvement remains unclear. Apical growth models for root hair and pollen tubes, for example, have been shown to share common features, although the dissimilar physiological functions, cell wall characteristics and growth environments need to be taken into account.

Several SI mechanisms depend on ROS pathways, and successful fertilization is likely to be achieved through Rboh isoforms capable of affecting pollen tube dynamics. These data also suggest that Rbohs are strongly involved in crosstalk between pollen and pistil. However, considerably less information has been collected on the generation of superoxide produced by NADPH oxidases in the gynoecium. Thus, unidentified female gametophyte-expressed Rboh(s) generate(s) ROS that can induce tube burst and sperm cell delivery, probably by means of cell wall weakening. A distinction needs to be drawn between this specialized mechanism and premature abnormal pollen tube rupture caused by the *rboh* mutant, which could be due to a previous increase in the rate of exocytosis whose cause is unknown. The integration of signaling pathways and known regulators could lead to the development of a model in which many remaining issues might be resolved. Moreover, despite the recent advances made, apart from the *Arabidopsis* model, little information is available regarding Rboh proteins in the reproductive tissues of plants. NADPH oxidase-dependent superoxide production in the reproductive tissues of species of agronomic value should be an important subject of study in the future.

AUTHOR CONTRIBUTIONS

MJ-Q, JT, and JA designed the review, collected the literature available and wrote the manuscript. All authors reviewed and approved the manuscript.

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MINI REVIEW
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Reactive Oxygen Species and Nitric Oxide Control Early Steps of the Legume – *Rhizobium* Symbiotic Interaction

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The symbiotic interaction between legumes and nitrogen-fixing rhizobium bacteria leads to the formation of a new organ, the nodule. Early steps of the interaction are characterized by the production of bacterial Nod factors, the reorientation of root-hair tip growth, the formation of an infection thread (IT) in the root hair, and the induction of cell division in inner cortical cells of the root, leading to a nodule primordium formation. Reactive oxygen species (ROS) and nitric oxide (NO) have been detected in early steps of the interaction. ROS/NO are determinant signals to arbitrate the specificity of this mutualistic association and modifications in their content impair the development of the symbiotic association. The decrease of ROS level prevents root hair curling and ITs formation, and that of NO conducts to delayed nodule formation. In root hairs, NADPH oxidases were shown to produce ROS which could be involved in the hair tip growth process. The use of enzyme inhibitors suggests that nitrate reductase and NO synthase-like enzymes are the main route for NO production during the early steps of the interaction. Transcriptomic analyses point to the involvement of ROS and NO in the success of the infection process, the induction of early nodulin gene expression, and the repression of plant defense, thereby favoring the establishment of the symbiosis. The occurrence of an interplay between ROS and NO was further supported by the finding of both S-sulfenylated and S-nitrosylated proteins during early symbiotic interaction, linking ROS/NO production to a redox-based regulation of the symbiotic process.

Keywords: legume, nitric oxide, nitrogen fixation, *Rhizobium*, symbiosis

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INTRODUCTION

Symbiosis describes a situation in which two or more organisms belonging to different species live together for an extended period of time (De Bary, 1879). Both partners can influence the fate of symbiosis, from the host side by the intensity of the immune response, and from the symbiont side by the degree of biological perturbations inflicted on the host (Moné et al., 2014). An important factor for the evolution of symbiosis is the control of redox environment (Hentschel et al., 2000). Redox homeostasis must be tightly controlled to stay under the situation of oxidative stress and to act as signaling pathway. The chemical instability of reactive oxygen species (ROS) and reactive nitrogen species in living organisms is an important property which explains their multi-faceted

roles in biology, particularly in plant–microbe interactions (Mittler et al., 2011; Puppo et al., 2013; Hichri et al., 2015; Meilhoc et al., 2015).

The symbiotic associations between Legumes (Fabaceae) and bacteria of *Rhizobium* type implies a recognition step which ultimately leads to the formation of nitrogen-fixing structures called nodules (Oldroyd and Downie, 2008). The interaction starts with the secretion of flavonoids by the plant roots. The perception of the flavonoids by the bacteria leads to the production of bacterial lipochito-oligosaccharides (Nod factors, NF), their specific recognition by the plant, and the induction of nodulation genes in both partners (Oldroyd and Downie, 2008). Increasing evidence support the critical role of ROS and nitric oxide (NO) in the recognition, signaling and immunity processes during the first steps of the symbiotic association between the two partners (Puppo et al., 2013). Both ROS and NO accumulate in roots and growing nodules according to specific spatiotemporal patterns, and regulate the expression of numerous genes that govern the development and the set-up of mature nodules. Several comprehensive reviews highlight the different functions played by these molecules in the nitrogen-fixing symbiosis (NFS) depending on the bacterial or plant origin (Boscari et al., 2013b; Puppo et al., 2013; Hichri et al., 2015; Meilhoc et al., 2015). In the present review, much attention will be paid on recent advance in the occurrence and the function of ROS and NO during the initial steps of NFS, and particularly on the specific role of NADPH oxidases, nitrate reductases (NRs) and hemoglobins (Hbs) in the control of the balance between ROS/NO production and catabolism.

ROLE OF ROS IN THE ESTABLISHMENT OF THE SYMBIOTIC INTERACTION

Involvement of NADPH Oxidase

Reactive oxygen species are transiently produced during rhizobial infection (Peleg-Grossman et al., 2009, 2012). The inhibition of ROS production by the NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI; Peleg-Grossman et al., 2007), and the correlation between ROS accumulation and transcript accumulation of two NADPH oxidase genes in response to NF in *Medicago truncatula* roots (Lohar et al., 2007), argue for the involvement of NADPH oxidases in ROS generation. NADPH oxidase genes, also named respiratory burst oxidase homologs (Rbohs), were recently identified and characterized in legume genomes (Lohar et al., 2007; Marino et al., 2011; Montiel et al., 2012). In *Phaseolus vulgaris*, nine *Rboh* genes have been identified (Montiel et al., 2012). *PvRbohB* is particularly accumulated in shoots, roots and nodules. Promoter activity of *PvRbohB* was detected during infection thread (IT) progression and nodule development. Transgenic roots knocked-down for *PvRbohB* by RNA interference (RNAi), Montiel et al. (2012) showed a reduced ROS production with concomitant reduction of nodulation. Microscopy analysis revealed that progression of the ITs was affected in *PvRbohB*-RNAi roots indicating that RbohB could play a key role in successful rhizobial colonization and proper IT growth and shape (Montiel et al., 2012). Ten *Rboh* genes were identified in *M. truncatula* genome (Marino et al., 2012). Their involvement in H_2O_2 synthesis during root infection by *Sinorhizobium meliloti* needs to be fully evidenced, but downregulation of legume *Rbohs* leads to decreased nodulation efficiency and an impairment of nitrogen fixation (Marino et al., 2011; Puppo et al., 2013).

The involvement of other potential enzymatic ROS sources cannot be excluded. Type III peroxidases (Prx-III), which were implicated in generation of apoplastic ROS are good candidates (Martinez et al., 1998; Bindschedler et al., 2006). They were reported to promote cell wall hardening (Passardi et al., 2004) and rigidification of IT cell wall and matrix (Wisniewski et al., 2000). Whole transcriptome analyses reveal that Prx-III genes, which were named *rhizobial induced peroxidases* (*Rip1-10*), are inducible by rhizobia and NFs in root hairs (Breakspear et al., 2014), suggesting that they could be involved in root hair ROS production during plant-symbiont recognition.

ROS in Root Hair Response to *Rhizobium* and NF

A rise of ROS was observed in root cortical cells of inoculated *M. truncatula* plants, which peaked at 24 h after inoculation and remained high after 48 h (Peleg-Grossman et al., 2009, 2012). During the infection process, production of superoxide anion (O_2^-) and H_2O_2 was localized in IT and infected cells (Santos et al., 2001; Ramu et al., 2002; Rubio et al., 2004). In *P. vulgaris,* a transient increase of ROS was detected at the tip of root hairs within seconds after NF addition (Cardenas et al., 2008). However, after several minutes H_2O_2 production appears to be inhibited by NF (Shaw and Long, 2003; Lohar et al., 2007). ROS production was not observed in *M. truncatula* plants inoculated with a *S. meliloti nodD1ABC* mutant unable to produce NF, indicating that the oxidative burst is activated downstream NF perception (Ramu et al., 2002). Furthermore, suppression of immune responses (ROS production, SA accumulation) was observed in *M. truncatula* and *M. sativa* roots upon addition of NF (Martínez-Abarca et al., 1998; Shaw and Long, 2003). It was suggested that ROS production is necessary for infection initiation, but prolonged and elevated levels are detrimental to nodulation (Toth and Stacey, 2015). The subsequent hypothesis is that NF may activate a first ROS production wave involved in nodule development, and inhibit a second one involved in defense reactions. The first wave would modulate the expression of plant genes and/or the redox status of proteins involved in root hair deformation (Lohar et al., 2007), IT progression and nodule formation (Montiel et al., 2012; Puppo et al., 2013).

Moreover, H_2O_2 appears to control a key step of the interaction. An *S. meliloti* strain, overexpressing a catalase gene, showed a delayed nodulation phenotype associated with aberrant IT (Jamet et al., 2007). The catalase overexpression probably decreased the internal H_2O_2 concentration of the bacteria progressing inside the IT, as observed in free-living conditions. Thus, a positive role for H_2O_2 during IT elongation was proposed that could be related to IT rigidity (Rathbun et al., 2002), or to a cytoplasmic signal used by the bacteria to regulate symbiotic function (Pauly et al., 2006). Alternatively, specific

posttranslational H_2O_2 protein modifications might occur in IT as observed for nitrogen fixing bacteroids (Oger et al., 2012).

ROLE OF NO IN THE ESTABLISHMENT OF THE SYMBIOTIC INTERACTION

NO Production

Several possible pathways of NO synthesis have been reported in plants which can be divided into oxidative (NO synthase like – NOS-like, polyamine-mediated, hydroxylamine-mediated) and reductive (NR, plasma membrane-bound nitrite NO reductase, mitochondrial electron transfer chain, xanthine oxidoreductase) pathways (Gupta et al., 2011; Mur et al., 2013). Two studies investigated potential NO source in the first step of NFS. In the first one, the treatment of soybean roots inoculated with *Bradyrhizobium japonicum* with the NOS inhibitor N_w-nitro-*L*-arginine (*L*-NNA) resulted in a 70% reduction of nodule number, suggesting the contribution of NOS-like enzyme in NO production (Leach et al., 2010). The other report shows that treatment of *M. truncatula* inoculated roots with tungstate, a NR inhibitor, mimics the addition of NO scavenger on the transcriptional regulation of genes involved in the nodulation process, whereas treatment with the NOS inhibitor L-NG-nitroarginine methyl ester (L-NAME) is ineffective (Boscari et al., 2013a). *M. truncatula* genome possess 3 NR genes, *MtNR3* being only expressed during the nodulation process (Puppo et al., 2013). *MtNR1* and *MtNR2* are strongly induced during nodulation process (**Figure 1**). These results suggest a specific role of these enzymes, as a NO source, during symbiosis establishment. The potential involvement of the other plant NO sources was not yet investigated.

In symbiotic bacteria, the main route for NO production is the denitrification pathway, which occurs both in free living bacteria under microoxic conditions and in nodules (Meilhoc

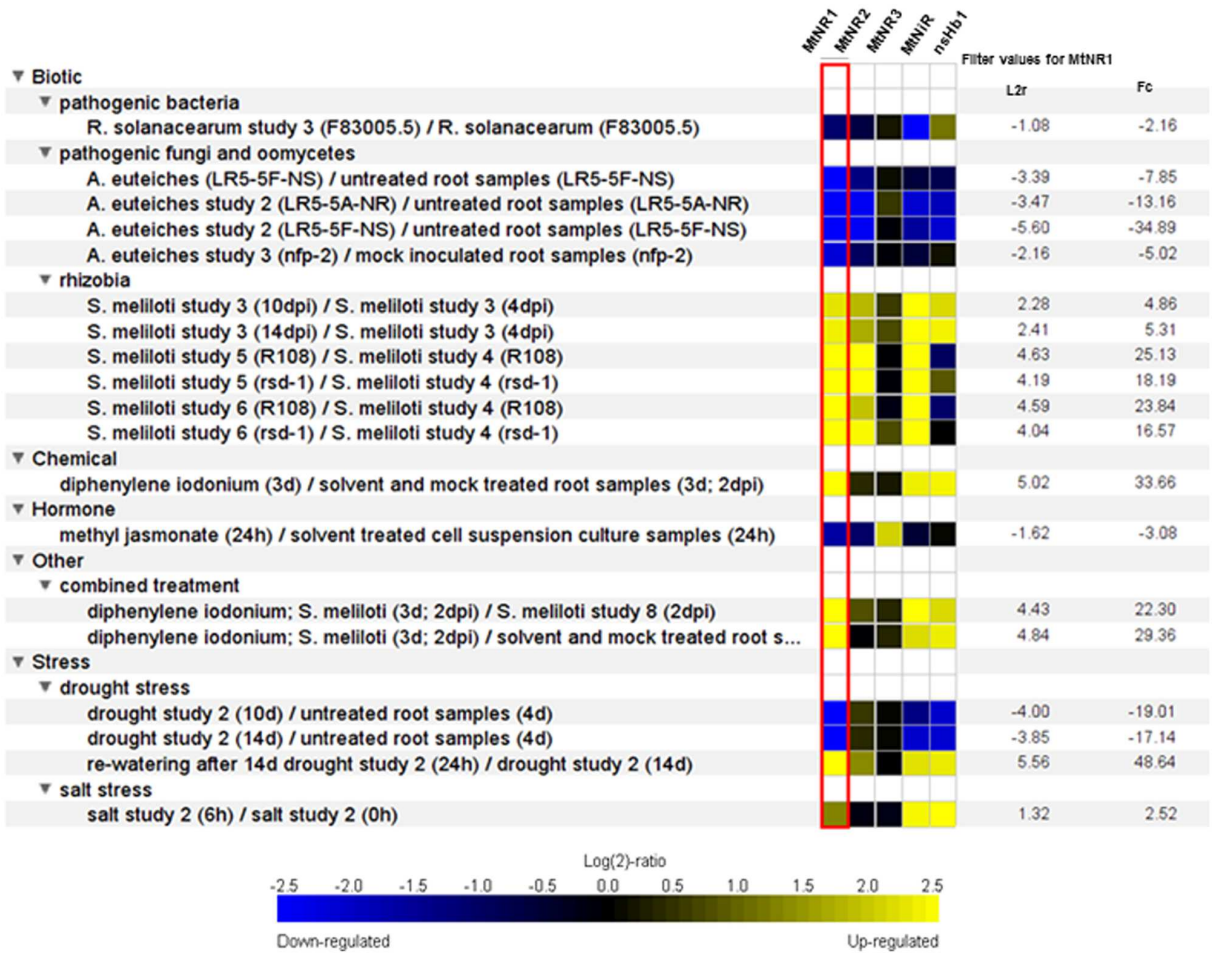

FIGURE 1 | Screenshot of the results obtained with the Genevestigator Perturbation tool for the *Medicago truncatula* MtNR1 (MTR_3g073170), MtNR2 (MTR_5g059820), MtNR3 (MTR_3g073150), MtNiR (MTR_4g086020), ns-Hb1 (MTR_4g068860) genes on the Affymetrix 61K Medicago GeneChip. The "Heatmap-List" view is presented here. Perturbations are ordered according to the expression log2-ratios of MtNR1 and are filtered to keep only the perturbations leading to a atleast twofold-change and a *p*-value of maximum 0.05. Perturbations having the highest log2 ratio are listed first. *R. solanacearum*, *Ralstonia solanacearum*; *A. euteiches*, *Aphanomyces euteiches*; *S. meliloti*, *Sinorhizobium meliloti*.

et al., 2011). The other way for NO production could be NOS-like enzymes which activity was reported in many bacteria (Sudhamsu and Crane, 2009). In *S. meliloti*, a L-arginine-dependent NO production was reported in free-living cells, although no corresponding gene was found in its genome (Pii et al., 2007). To date, there is no evidence for an involvement of the bacterial partner in NO production during symbiosis establishment.

NO Metabolism

Toxic or signaling effects of NO depend on its concentration at the site of action (Mur et al., 2013). Therefore, NO concentration should be regulated to allow signaling functions to occur and to limit toxic effects. Hbs are known to act as NO scavengers in plants (Gupta et al., 2011). Based on their sequence homology and affinity for O_2, plant Hbs have been classified into non-symbiotic hemoglobins (ns-Hbs), leghemoglobins, (Lbs), and truncated hemoglobins (tr-Hbs) (Gupta et al., 2011; Hill, 2012). The three Hb types are expressed in legumes (Nagata et al., 2008; Bustos-Sanmamed et al., 2011). Due to their very high affinity for O_2 and NO (*Km* in the range of 1–20 nM), ns-Hbs and Lbs are able to scavenge O_2 and NO traces to convert them to nitrate (Hill, 2012). Lbs, which accumulate to millimolar concentration in infected nodule cells (Appleby, 1992), are thought to buffer free O_2 in the nanomolar range, avoiding inactivation of nitrogenase while maintaining high O_2 flux for respiration (Ott et al., 2005). Tr-Hbs are induced in *M. truncatula* (Vieweg et al., 2005), and *Frankia* spp. (Niemann and Tisa, 2008; Coats et al., 2009) during NFS. Based on their expression pattern, it was also suggested that they could be involved in NO homeostasis. Other NO metabolizing pathways such as *S*-nitrosoglutathione reductase (GSNOR), glutathione peroxidase and thioredoxin systems, which are known to also regulate NO level in plants (Leterrier et al., 2011; Correa-Aragunde et al., 2015) have been evidenced in legumes (Renard et al., 2011; Matamoros et al., 2015), but their respective contributions to NO balance remains to be investigated in NFS.

Three Hb classes have been also described in bacteria: flavo-Hb (Hmp), single-domain Hb (sd-Hb), and truncated Hb (tr-Hb), which exhibit NO scavenging and detoxification activity (Sanchez et al., 2011). The lower competitiveness of *S. meliloti* overexpressing *hmp* strains as compared to the WT is an argument in favor of a Hmp role in NO control during the infection process (del Giudice et al., 2011). The respiratory NO reductase (Nor) and two proteins of the NnrS family (NnrS1 and NnrS2) were shown to be also involved in NO degradation and to be essential in maintaining efficient NFS (Cam et al., 2012; Meilhoc et al., 2013; Blanquet et al., 2015). However, their role in NO control during symbiosis establishment was not yet investigated.

NO in the Recognition of *Rhizobium* Partner

Numerous findings support the hypothesis that NO signaling plays a role in plant microbe recognition. Treatments of soybean roots with L-NNA reduced nodule number during interaction with *B. japonicum*, a phenotype reverted by the addition of the NO-donor diethylenetriamine NONOate (Leach et al., 2010). In the same way, the decrease of the NO content by 2-[4-carboxyphenyl]-4,4,5,5-tetramethyl imidazoline-1-oxyl-3-oxide (cPTIO) treatment and by *hmp* overexpression in the plant partner delayed nodulation in the *M. truncatula* – *S. meliloti* interaction, indicating that NO is required for an optimal establishment of the symbiotic process (del Giudice et al., 2011). Several reports underlined the role of plant Hbs in regulating NO level during symbiosis establishment. First, upon *Lotus japonicus* inoculation with *Mesorhizobium loti*, a transient production of NO was observed at the root surface 4 h post-inoculation (hpi), which then decreased to its basal level 10 hpi (Nagata et al., 2008). However, when *L. japonicus* was infected with the plant pathogens *Ralstonia solanacearum* and *Pseudomonas syringae*, NO was continuously produced for at least 24 hpi (Nagata et al., 2008). The decrease in NO level after its transient accumulation following infection with *M. loti* was assigned to *LjHB1* which gene expression was upregulated by the symbiont, but not by the pathogens (Nagata et al., 2008, 2009; Murakami et al., 2011). In addition, NO was shown to up-regulate ns-Hb expression in different plant species (Shimoda et al., 2005; Sasakura et al., 2006; Nagata et al., 2008). These observations suggest that at early step of symbiotic interaction, the initial burst of NO induces the expression of ns-Hb that, in return, scavenges NO and down-regulates its level to lower plant defense response and allow the reception of the symbiont. Experimental evidence showed that lipopolysaccharides from *M. loti* induced *LjHB1* expression and NO production in *L. japonicus* roots (Nagata et al., 2009; Murakami et al., 2011). They showed the polymer distributed on the outer membrane of Gram-negative bacteria play a major role in the recognition and establishment of symbiosis.

Nitric oxide production was also observed in the infection pockets, along the ITs, and in dividing cortical cells of the nodule primordia (del Giudice et al., 2011). The presence of NO in nodule primordia present high similarity with the local NO increase observed in lateral root primordia (Correa-Aragunde et al., 2004; Lanteri et al., 2006). In both studies, authors reported that NO could modulate the expression of cell cycle regulatory genes (Correa-Aragunde et al., 2006; del Giudice et al., 2011). It was notably observed in *M. truncatula* that NO scavenging provokes the down regulation of plant genes involved in nodule development, such as *MtCRE1* and *MtCCS52A* (del Giudice et al., 2011). Furthermore, transcriptomic analysis of cPTIO-treated inoculated roots of *M. truncatula* showed that NO is involved in the regulation of many family of genes related to cell cycle process and protein synthesis in nodule primordia (Boscari et al., 2013a), which reinforces the hypothesis that NO plays a similar function in nodule and lateral root organogenesis.

Interestingly, the control of NO homeostasis through the spatiotemporal coordination of NR and Hb gene expression was recently hypothesized to participate to nitrate sensing in maize roots (Trevisan et al., 2015). Moreover, Frungillo et al. (2015) demonstrate that NO is at the center of nitrogen homeostasis in *Arabidopsis* plants. They demonstrated that NO derived from nitrate assimilation inhibits the activity of GSNOR, which controls the cellular levels of GSNO, by *S*-nitrosylation (addition

of a NO group to cysteine thiols) of some of its cysteine residues. They observed that inhibition of GSNOR is necessary to amplify a SNO signal, which in turn feedback regulates nitrate assimilation (**Figure 2**). It is noteworthy that nodulation efficiency is as well finely tuned according to the nitrate availability (Streeter, 1988).

NO AND ROS INTERPLAY

Overall, it appears that ROS and NO present a fine-tuned spatio-temporal modulation which plays a critical role in signaling and immunity in the associations between Legumes and rhizobia. The occurrence of an interplay between ROS and NO was supported by the finding of both *S*-sulfenylated and *S*-nitrosylated proteins posttranslational modification during NFS (Oger et al., 2012; Puppo et al., 2013), linking ROS/NO production to a redox-based regulation of the symbiotic process. Peroxynitrite ($ONOO^-$) is a signaling molecule formed when NO reacts with O_2^-. Its function may be mediated by the selective nitration of Tyr residues in a small number of proteins. Blanquet et al. (2015) reported that glutamine synthetase GS1a, a key enzyme in N_2−fixation, is nitrated in nodules. Such NO/$ONOO^-$-mediated posttranslational inactivation of GS provides a direct link between NO/O_2^- signaling and nitrogen metabolism in root nodules. Whether these molecules work in synergy in early symbiosis steps is not determined yet, however, the comparison of *M. truncatula* inoculated roots treated with either the NADPH oxidase inhibitor (Andrio et al., 2012), or the NO scavenger (Boscari et al., 2013a), reveals a strong overlap in the signaling pathways triggered by either molecule. Furthermore 75% of the 316 differentially regulated genes identified in both analyses are similarly up- or down-regulated (Puppo et al., 2013). Amongst the up-regulated genes, some gene families involved in plant defense and secondary metabolism were notably identified, as previously highlighted during cell death induction (Zago et al., 2006). Moreover, in transcriptomic analysis of DPI-treated *M. truncatula* roots (Andrio et al., 2012), *MtNR1*, *MtNiR,* and *Mtns-Hb1* were found strongly up-regulated (**Figure 1**), suggesting that H_2O_2 could control the transcriptional regulation of enzymes involved in NO homeostasis. In the same way, it was recently reported that an elevated ROS concentration during plant–pathogen interaction results in the activation of NR (Wang et al., 2010; Lin et al., 2012). Interestingly, in *Arabidopsis* leaves, NR activation leads to increased NR-mediated NO production, and to the subsequent inactivation of NADPH oxidase activity by *S*-nitrosylation during pathogen infection (Yun et al., 2011). These results evidence the overlapping connection between NO and ROS production with a negative feedback loop of the NO

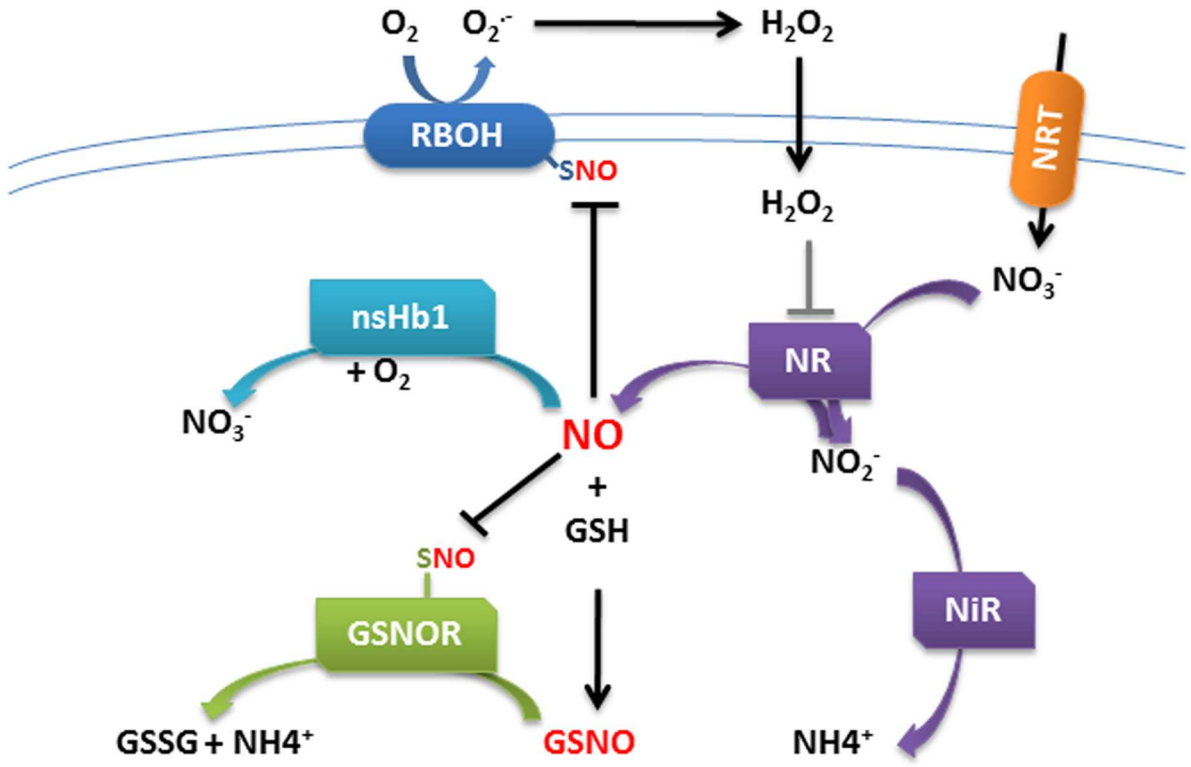

FIGURE 2 | Schematic model of cross-talk regulation between ROS and NO in plant cells. H_2O_2 produced by NADPH oxidase leads to the activation of nitrate reductase (NR) and concomitant NO production. NO accumulation in turn blunts NADPH oxidase activity by *S*-nitrosylation, preventing accumulation of excess ROS. In the nitrogen assimilation pathway, nitrate (NO_3^-) is taken up by nitrate transporters (NRT) and reduced to nitrite (NO_2^-) by NR. Nitrite is reduced to NO by NR. NO reacts with reduced glutathione (GSH) in the presence of O_2 to form *S*-nitrosoglutathione (GSNO). GSNO is converted by the enzyme GSNO reductase (GSNOR) into oxidized glutathione (GSSG) and NH_3. NO accumulation leads to GSNOR *S*-nitrosylation and inhibition preventing GSNO degradation.

on ROS production (**Figure 2**). However, such type of ROS/NO interplay still has to be clearly elucidated in roots and during establishment of NFS.

CONCLUSIONS AND FUTURE DIRECTIONS

We showed that spatio-temporal accumulations of ROS and NO are critical for the specificity of their function throughout the successive steps of symbiosis initiation. NADPH oxidases were identified as major source of ROS production, and NR and NOS-like have been evidenced as NO sources during the early steps of the interaction. Importance of plant and bacterial Hbs in NO balance was particularly highlighted. However, the involvement of alternative systems like catalase, glutathione peroxidase, GSNOR and thioredoxins remains to be investigated to decipher their respective contribution in ROS and NO balance. Transcriptomic analyses point to the involvement of ROS and NO in the success of the infection process notably by the repression of plant defense responses favoring the establishment of the symbiosis Further investigations will aim to decipher the possible regulation of nodule NADPH oxidase activity by NO and the transcriptional regulation of genes involved in NO homeostasis by H_2O_2 in symbiosis establishment.

AUTHOR CONTRIBUTIONS

ID, NP, and AP contribute to the writing of the different paragraph about ROS. RB and AB contribute to the writing of the different paragraph about NO and interplay ROS/NO.

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Cross-Regulation between N Metabolism and Nitric Oxide (NO) Signaling during Plant Immunity

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Plants are sessile organisms that have evolved a complex immune system which helps them cope with pathogen attacks. However, the capacity of a plant to mobilize different defense responses is strongly affected by its physiological status. Nitrogen (N) is a major nutrient that can play an important role in plant immunity by increasing or decreasing plant resistance to pathogens. Although no general rule can be drawn about the effect of N availability and quality on the fate of plant/pathogen interactions, plants' capacity to acquire, assimilate, allocate N, and maintain amino acid homeostasis appears to partly mediate the effects of N on plant defense. Nitric oxide (NO), one of the products of N metabolism, plays an important role in plant immunity signaling. NO is generated in part through Nitrate Reductase (NR), a key enzyme involved in nitrate assimilation, and its production depends on levels of nitrate/nitrite, NR substrate/product, as well as on L-arginine and polyamine levels. Cross-regulation between NO signaling and N supply/metabolism has been evidenced. NO production can be affected by N supply, and conversely NO appears to regulate nitrate transport and assimilation. Based on this knowledge, we hypothesized that N availability partly controls plant resistance to pathogens by controlling NO homeostasis. Using the *Medicago truncatula/Aphanomyces euteiches* pathosystem, we showed that NO homeostasis is important for resistance to this oomycete and that N availability impacts NO homeostasis by affecting *S*-nitrosothiol (SNO) levels and *S*-nitrosoglutathione reductase activity in roots. These results could therefore explain the increased resistance we noted in N-deprived as compared to N-replete *M. truncatula* seedlings. They open onto new perspectives for the studies of N/plant defense interactions.

Keywords: nitrogen metabolism, plant immunity, *Aphanomyces euteiches*, *Medicago truncatula*, nitric oxide homeostasis

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NITROGEN AND THE PLANT IMMUNE RESPONSE

Plants are under the constant threat of pathogen attacks that limit their survival and are major yield-limiting factors. In response to these attacks, plants activate multiple defense reactions both at the site of infection and systemically, which in many cases lead to resistance. These reactions include massive transcriptional reprogramming, cell wall reinforcement, synthesis of

antimicrobial metabolites, and production of pathogenesis-related (PR) proteins. These events are mediated by a variety of rapidly mobilized molecules, such as second messengers, e.g., Ca^{2+}, protein kinases, reactive oxygen species (ROS), or reactive nitrogen species (RNSs), including nitric oxide (NO). Although these defense responses have been widely studied, it has become increasingly obvious over the past years that a plant's capacity to mobilize them is greatly affected by its physiological status (Snoeijers et al., 2000) and its development (Develey-Riviere and Galiana, 2007).

Nutrients are important for the growth and development of plants and microorganisms. Among them, nitrogen (N) can affect the fate of an interaction between a plant and a pathogen (Dordas, 2008). No general rules can be drawn about modification of resistance by N. Although we know that N lack or excess, along with the nature of available N in soil, can modulate plant resistance (Huber and Watson, 1974), the underlying mechanisms remain unclear. Recent works indicate that plants' capacity to acquire and assimilate N could partly explain nutrition effects on plant defense. N is taken up by the roots mostly in the form of nitrate (NO_3^-) in aerobic soils and ammonium (NH_4^+) in flooded wetlands or acidic soils. Ammonium taken up directly from the soil or resulting from the reduction of NO_3^- and nitrite (NO_2^-) by nitrate reductase (NR) and nitrite reductase (NiR), respectively, is assimilated *via* the glutamine synthetase (GS)/glutamate synthase cycle (Xu et al., 2012). The uptake of mineral N from the soil and the subsequent distribution to the whole plant is driven by nitrate transporters from the multigenic *NRT2* and *NPF* families and by ammonium transporters from the *AMT* family (Krapp, 2015). The contribution of several of these transporters to plant defense has recently been highlighted in *Arabidopsis thaliana*. For instance, induction of *AMT1.1* expression was evidenced upon infection by the bacterium *Pseudomonas syringae* or the fungus *Erysiphe cichoracearum* (Liu et al., 2010). The role of specific transporters was demonstrated using plant mutants: *nrt2* (deficient in the expression of the *NRT2.1* and *NRT2.2* genes) and *nrt2.6-1* mutants displayed altered sensitivity to the bacterial phytopathogens *P. syringae* and *E. amylovora* (Camanes et al., 2012; Dechorgnat et al., 2012). Besides N uptake into plants and its subsequent allocation, several results indicate that N assimilation and particularly amino acid homeostasis can impact plant-pathogen interactions (Zeier, 2013; Luna et al., 2014). Conversely, pathogen attacks are correlated with modulation of the expression of genes or of the activity of enzymes involved in N assimilation such as NR or GS2, in N remobilization such as GS1, and in amino acid metabolism [reviewed by Fagard et al. (2014)]. Whether these changes in N metabolism reflect the manipulation of host metabolism by the pathogen or result from the modulation of plant defenses is not always clear. Interestingly, some members of the GLR glutamate receptor family were recently proposed to play a role as amino acid sensors during plant defense, perhaps by sensing changes in extracellular amino acids caused by pathogen infection (Forde and Roberts, 2014). Crosstalk between N metabolism and phytohormones can also interfere with plant stress responses and could be considered as a mechanism involved in the partitioning of available resources between defense and growth. For instance, N limitation induced the accumulation of salicylic acid (SA) in *A. thaliana* leaves (Yaeno and Iba, 2008). Conversely, ethylene/jasmonic acid signaling coordinated the upregulation of the nitrate transporter *NRT1.8* (*AtNPF7.2*) and the downregulation of *NRT1.5* (*AtNPF7.3*) genes to tune NO_3^- reallocation in plants from the shoot to the roots under stress conditions (Zhang et al., 2014). Finally, experiments on rice showed that N-induced susceptibility to *Magnaporthe oryzae* is genotype-dependent, and may be linked to N use efficiency (Ballini et al., 2013). These interesting data raise the question of the genetic control of N effects on plant immunity. The identification of the corresponding QTLs will permit to uncover new molecular actors of N-controlled resistance to pathogens.

NITRIC OXIDE AND N METABOLISM

The role of NO in plant defense is widely accepted. NO is involved in transcriptional regulation of defense genes encoding PR proteins or proteins involved in phytoalexin synthesis, SA accumulation, and post-translational protein modifications (Wendehenne et al., 2014). NO is a nitrogen species produced *via* a variety of pathways in plants (reviewed by Gupta et al., 2011c). Briefly, these pathways can be classified into two groups according to nitrogen-containing precursors: the L-arginine-dependent pathway (oxidative pathway), and the NO_2^--dependent pathway (reductive pathway). NO_2^--dependent NO synthesis involves NR which reduces NO_2^- to NO both *in vitro* and *in vivo* in specific physiological contexts (Yamasaki and Sakihama, 2000); alternatively, formation of NO through the reduction of NO_2^- by the mitochondrial respiratory chain can also be observed, particularly in roots (Gupta et al., 2011a; Horchani et al., 2011). Finally, NO can be produced by an apoplastic non-enzymatic conversion of NO_2^- to NO at acidic pH, in the presence of reductants such as ascorbic acid (Bethke et al., 2004).

Several pathways involved in NO transformation and turnover and balancing the bioavailability of this molecule have been identified (Leitner et al., 2009). Firstly, NO can react with reduced glutathione to produce *S*-nitrosoglutathione (GSNO), a low-molecular-weight *S*-nitrosothiol (SNO) that is more stable than NO and considered to be a mobile reservoir of NO. The cellular level of GSNO is enzymatically regulated primarily by GSNO reductase (GSNOR), which catalyzes the reduction of GSNO to oxidized glutathione and ammonium. Importantly, Yun et al. (2016) recently reported that NO and GSNO have additive functions in plant immunity but also in plant development. NO and GSNO might have distinct or overlapping molecular targets, thus allowing differential control of key cellular processes belonging to both defense and development. Secondly, besides their O_2 binding properties, hemoglobins (Hbs) can metabolize NO into NO_3^- and therefore are also considered as NO and NO_2^- concentration modulators (Gupta et al., 2011b). Finally, NO rapidly reacts with superoxide ($O_2^{\cdot-}$) to

form peroxynitrite ($ONOO^-$), an oxidizing and nitrating RNS produced for instance in plant cells during immune responses (Vandelle and Delledonne, 2011). These molecules associated with NO turnover also play a role in the plant immune response. For instance, GSNO plays a key role in mediating the structural and functional changes of NPR1, a key transcription coactivator of plant immunity (Tada et al., 2008).

Nitric oxide is partly produced through NR, dependent on its substrate/product NO_3^-/ NO_2^- as well as on L-arginine and polyamines. As a result, cross-regulation between NO signaling and N supply/metabolism is expected. Several lines of evidence show that NO production is likely to be affected by N supply. In a physio-pathological context, plant NO production is dependent on the form of N supply. Besson-Bard et al. (2008) and Gupta et al. (2013) showed that tobacco cell suspensions or leaves from plants grown on ammonium instead of nitrate as an N source emitted less NO when elicited by cryptogein or *P. syringae*. Thus these data highlight the determining role of the N source on the rate of NO synthesis. Modifications of the intracellular concentration of diverse intermediates of N metabolism such as amino acids or polyamines also result in the modulation of NO production. For instance, exogenously added polyamines induced rapid NO biosynthesis in *A. thaliana* (Tun et al., 2006). In the same manner, overexpression of the Asparagine synthetase 1 gene significantly enhanced the NO burst (Hwang et al., 2011). Finally, N nutrition could also impact important redox molecules associated with NO homeostasis. Nitrate deprivation led to altered levels of ROSs in *A. thaliana* and tobacco (Schachtman and Shin, 2007; Besson-Bard et al., 2008). Pathogen-induced expression of the nitrate transporter *NRT2.6* was also correlated with ROS accumulation (Dechorgnat et al., 2012). Concentrations of antioxidant molecules such as glutathione (GSH) were altered (decreased in shoots and increased in roots) in *A. thaliana* and barley plants exposed to N deficiency (Kandlbinder et al., 2004; Kovacik et al., 2014).

Reciprocally, NO and derived RNS could participate in the regulation of N metabolism. NO can control physiological processes by modifying gene transcription. By analyzing available literature and databases, we identified interesting candidates likely to contribute to the crosstalk between N metabolism and NO among the numerous NO-regulated genes. Transcriptomic studies highlighted the up- or down-regulation of transcripts encoding N transporters (Ahlfors et al., 2009; Corti Monzon et al., 2014; Trevisan et al., 2015) or N assimilation/remobilization genes (Ferrarini et al., 2008; Ahlfors et al., 2009; Xu et al., 2013; Begara-Morales et al., 2014; Corti Monzon et al., 2014; Zeng et al., 2014; Trevisan et al., 2015) and amino acid metabolism-related genes (Ferrarini et al., 2008; Xu et al., 2013) upon modulation of NO homeostasis by treatment with NO donors, NO scavengers, or using mutants affected in NO homeostasis. Physiological studies identified NO as a regulator of N uptake in *Chlamydomonas reinhardtii*, possibly through the control of the expression of the nitrate or ammonium (*AMT1.1 and AMT2.2*) transporters. In *A. thaliana,* the expression of the high affinity nitrate transporter *NRT2.1* was down-regulated by NO donors and in a *GSNOR* knock-out mutant, but the expression of the low-affinity nitrate transporter *NRT1.1* remained unaltered (Frungillo et al., 2014), suggesting a switch from high- to low-affinity nitrate transport. By contrast, the expression of *NRT2.1* was up-regulated through an NO-dependent process in *A. thaliana* roots exposed to cadmium (Besson-Bard et al., 2009). In addition to NO-mediated transcriptional regulation, many of NO biological functions arise as a direct consequence of chemical reactions between proteins and NO/RNS. Metal-nitrosylation, *S*-nitrosylation, and tyrosine nitration are notably emerging as main NO-dependent post-translational protein modifications (Astier and Lindermayr, 2012). Among the soluble proteins identified as *S*-nitrosylated or Tyr-nitrated, possible candidates contributing to the NO/N metabolism interplay are mainly involved in both N assimilation/remobilization and amino acid metabolism (**Table 1**). Post-translational inhibition of high-affinity ammonium and high-affinity NO_3^-/ NO_2^- transporters by NO was highlighted in *C. reinhardtii* (Sanz-Luque et al., 2013). However, whether the reversible effect of NO was linked to *S*-nitrosylation of the transporters or to an indirect effect of NO leading to other post-translational modifications of the transporters remains to be determined (Sanz-Luque et al., 2013). In that same study, NO also inhibited NR activity reversibly, but not NiR or GS activity. This post-translational effect of NO on N transporters and NR might mediate the fast inhibition of N uptake and assimilation by ammonium in *C. reinhardtii*. More recently, inhibition of NR activity by NO was proposed to be partly mediated by a truncated hemoglobin THB1 whose gene expression is highly induced by NO (Sanz-Luque et al., 2015).

In higher plants, NO produced by denitrification in the rhizosphere of forest soils impacts N uptake without affecting gene expression patterns of putative N transporters, suggesting post-translational modification of these transporters (Dong et al., 2015). NR is also highly regulated by complex transcriptional and post-translational mechanisms. Studies on different models using NO donors, NO synthase inhibitors, or the scavenger cPTIO indicate that NO modulates NR activity. Results are sometimes contradictory. NR activity in leaves was inhibited under high NO concentrations (Rosales et al., 2011, 2012; Frungillo et al., 2014), but was enhanced in cabbage (Du et al., 2008). Moreover, the inhibition or activation of NR by NO in tomato roots could depend on the NO_3^- concentration (Jin et al., 2009). The mechanisms explaining these effects of NO on NR are poorly understood. Regulation of NR by NO could occur through transcriptional downregulation of the NR *NIA* genes in *Chlamydomonas* and *A. thaliana* (de Montaigu et al., 2010). A direct interaction of NO with NR is possible, as *S*-nitrosylation of NR was evidenced in poplar exposed to cold stress (Cheng et al., 2015). Glutamine synthetase 2 is a second key enzyme of plant N metabolism involved in the synthesis of essentially nitrogenous compounds *via* Gln production. Interestingly, GS1 and GS2 were identified as molecular targets of NO (**Table 1**). GS activity was inhibited by Tyr nitration in root nodules of *Medicago truncatula*. This post-translational modification may mediate channeling of glutamate to boost plant antioxidant defenses (Melo et al., 2011) in response to NO. This interesting feature does not seem to be shared across the plant kingdom

TABLE 1 | Examples of *S*-nitrosylated or Tyr-nitrated proteins involved in N and amino acid metabolism.

Functions	Post-translational modifications	Identified Proteins	Conditions	Reference
Amino acid metabolism	Tyrosine nitration	Methionine synthase	–	Lozano-Juste et al., 2011
	S-nitrosylation	Asparagine synthase 3	Biotic stress	Maldonado-Alconada et al., 2011
		Glutamate decarboxylase	Biotic stress	Maldonado-Alconada et al., 2011
		EPSP synthase	Biotic stress	Astier et al., 2012
		Acetohydroxy acid isomeroreductase (Val and Ile synthesis)	Biotic stress	Astier et al., 2012
		Aspartate aminotransferase	Biotic stress	Astier et al., 2012
		Cysteine synthase	Abiotic stress	Puyaubert et al., 2014
		Alanine glyoxylate aminotransferase	Abiotic stress	Puyaubert et al., 2014
		Glutamate glyoxylate aminotransferase	Abiotic stress	Puyaubert et al., 2014
Nitrogen metabolism	Tyrosine nitration	Glutamine synthetase 2	Biotic stress	Cecconi et al., 2009; Lozano-Juste et al., 2011
		Glutamine synthetase 1	Rhizobium-legume symbiosis	Melo et al., 2011
	S-nitrosylation	Argininosuccinate synthase	Biotic stress	Maldonado-Alconada et al., 2011
		Nitrite reductase	*atgsnor1–3*	Hu et al., 2015
		Glutamate synthase	Abiotic stress	Puyaubert et al., 2014
		Glutamate dehydrogenase 1	Biotic stress	Maldonado-Alconada et al., 2011
		Glutamate dehydrogenase 2	Biotic stress	Maldonado-Alconada et al., 2011

since GS activity was not affected by the NO donor DEA-NONOate in the alga *Chlamydomonas* (Sanz-Luque et al., 2013).

ROLE OF NO/RNS IN THE MODULATION OF THE IMMUNE RESPONSE BY N NUTRITION: FIRST EXPERIMENTAL EVIDENCE

Altogether, these data indicate that N supply has an impact on plant immunity and NO/RNS signaling and lead us to wonder about the role of NO/RNS in the modulation of the immune response by N nutrition. In the present work, we used an *in vitro* pathosystem composed of the legume *M. truncatula* challenged with the soil-borne root pathogen *Aphanomyces euteiches*. This oomycete is considered as the most limiting factor for legume production. Resistance of *M. truncatula* roots includes protection of the central cylinder against pathogen invasion, associated with frequent pericycle cell divisions, lignin deposition, and accumulation of soluble phenolic compounds (Djébali et al., 2009). First investigations of the biochemical processes underlying the expression of this resistance showed modulation of H_2O_2 levels and of the activity of antioxidant enzymes (Djébali et al., 2009, 2011). Interestingly, in the *M. truncatula* A17 genotype, resistance against *A. euteiches* was significantly enhanced in response to NO_3^- starvation as compared to sufficient N conditions (Thalineau et al., unpublished). Based on the current literature, we hypothesized that NO could play a role in this N-induced modulation of *M. truncatula* defense responses against *A. euteiches*. We therefore first assessed whether changes in NO homeostasis could indeed affect *M. truncatula* resistance to *A. euteiches*. Secondly, we determined whether NO homeostasis could be modulated by N nutrition during the *M. truncatula-A. euteiches* interaction. We considered NO homeostasis as the maintenance of a functional NO concentration in a specific condition, through a balance between its biosynthesis (e.g., NR activity) and turnover pathways (e.g., interactions with GSH or $O2^{\cdot-}$ to form GSNO or $ONOO^-$, respectively).

MATERIALS AND METHODS

Plant Growth and Inoculation by *A. euteiches*

We used the *M. truncatula* Jemalong-A17 genotype. *M. truncatula* seeds were scarified according to Djébali et al. (2009). After stratification overnight at 4°C, they were germinated in phytochambers with 16 h light under 350 µmol m^{-2} s^{-1} photons at 23°C /8 h night at 21°C. One day after germination, the seedlings were transferred to 12 cm × 12 cm square Petri dishes containing modified M medium (Bécard and Fortin, 1988). This modified medium was sugar-free, enriched in phosphate (1.3 mM final concentration), and contained either 3.2 mM nitrate (complete medium) or no nitrate (NØ medium). The Petri dishes were sealed with parafilm and the roots were protected from light with aluminum foil, and then placed vertically in the culture chamber (16 h light under 350 µmol m^{-2} s^{-1} photons at 23°C/8 h night at 21°C) for 7 days. The strain *Aphanomyces euteiches* Drechs ATCC 201684 was used to inoculate the seeds one day after germination. Zoospores were produced as described in Rey et al. (2013), and each root was inoculated with 500 zoospores.

Agrobacterium rhizogenes Root Transformation

The pENTR4 vector carrying the MtNR1 or the MtNR2 fragment (Horchani et al., 2011) was recombined with the pK7GWIWG2d vector using LR clonase II enzyme mix (Invitrogen, France) to create RNA interference expression vectors. The MtGSNOR gene (*M. truncatula* Gene code Medtr7g099040) (1,143 bp) was amplified using *M. truncatula* cDNA as a template and the specific primers GSNOR-F 5′-AAAAAGCAGGCTTCACCATGGCATCGTCGACTGAAGGT-3′ and GSNOR-R 5′- AGAAAGCTGGGTGTCAATGCAATGCAAGCACAC containing the corresponding attB recombination sites. The PCR product was recombined into the pDONR entry vector (Invitrogen) and checked by sequencing. The pDONR vector carrying the MtGSNOR gene was recombined with pK7WG2d plasmids[1] to create the overexpression vector. The constructs pK7GWIGW2d-MtNR1-2/GFP (RNAi::*MtNIA1/2*) and pK7WG2d-MtGSNOR/GFP (35S::GSNOR) were introduced into *A. rhizogenes* strain Arqua1 (Quandt et al., 1993). *M. truncatula* plants were transformed with *A. rhizogenes* according to Boisson-Dernier et al. (2001). Control plants were transformed with *A. rhizogenes* containing the pK7GWIGW5D or the pK7WG2d empty vectors. Hairy roots were selected based on the fluorescent marker GFP 21 days after transformation.

RNA Extraction, Reverse Transcription, and Quantitative PCR on Transformed Roots

Total RNA was extracted from transformed roots using TRIzol® Reagent (Life Technologies) according to the manufacturer's recommendations. To carry out the qPCR reaction, RNAs (0.5–1 μg) were reverse-transcribed in a final volume of 20 μL in the presence of RNasin (Promega, Charbonnières, France), and oligo(dT)$_{15}$, with M-MLV reverse transcriptase (Promega, Charbonnières, France), as recommended by the manufacturer.

Quantitative PCR was performed on reverse-transcribed RNAs from four independent biological replicates per condition and from two independent plant cultures. Quantitative PCR reactions were performed in an ABI PRISM 7900 sequence detection system (Applied Biosystems®, Saint-Aubin, France), in a final volume of 15 μL containing Absolute SYBR green ROX Mix (Thermo Scientific, Surrey, UK), 0,3 μM of gene-specific primers, and 5 μL of cDNA template diluted 60-fold. The reference gene used for normalization was *MtEF1α*. Relative expression was expressed as $2^{-\Delta Ct\ \text{test genes}-\text{reference gene}}$. The primers used for the qPCR all displayed a high amplification efficiency (90–100%). They were the following:

MtGSNORforward 5′-GTGACTGGGCGTGTATGGAA-3′
MtGSNORreverse 5′-TGCAAGCACACAACGAAGAC-3′
MtNIA1forward 5′-TGTTCCACAGGCTTCTCCAGATACA-3′
MtNIA1reverse 5′-CATACAGCGTCGTACTCAGCGACA-3′
MtNIA2forward 5′GCAAACCGGACGGAGGATGA-3′
MtNIA2reverse 5′CCGTGATGAATCCCACACTATATTCC-3′
MtEF1αforward 5′-AAGCTAGGAGGTATTGACAAG-3′
MtEF1αreverse 5′-ACTGTGCAGTAGTACTTGGTG-3′

[1]https://gateway.psb.ugent.be/

Inoculation of Transformed Root Cultures with *A. euteiches*

Roots were cultured on Shb10 medium (Boisson-Dernier et al., 2001) and transferred on modified Fahraeus medium enriched in ammonitrate (1 mM NH_4NO_3 final) one day before inoculation. Inoculation of the root cultures with *A. euteiches* strain ATCC 201684 was carried out by adding 10 mL of an *A. euteiches* zoospore suspension containing 80,000 zoospores.mL^{-1} in sterilized Volvic (Colditz et al., 2007) water. Zoospore production was initiated as described in Rey et al. (2013). Control root cultures were inoculated with 10 mL of sterile Volvic water. After 4 h of incubation in the dark, the zoospore solution was drained off the roots, and the Petri dishes were placed back into the growth room and left there for 7 days in the dark.

Assessing Infection Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

Assessment of *A. euteiches* development in roots was performed by ELISA, using rabbit polyclonal serum raised against *A. euteiches*, and a mouse anti-rabbit IgG alkaline phosphatase conjugate as described by Slezack et al. (1999), on protein extracts from roots from pooled plants. Alkaline phosphatase activity was monitored by recording the increase in absorbance at 405 nm for 2–3 h, and was expressed as the slope of the resulting curve per mg of root fresh weight.

Hydrogen Peroxide Quantification

H_2O_2 concentration was measured using an Amplex Red®/peroxidase-coupled fluorescence assay adapted from Ashtamker et al. (2007). Roots were ground on ice and in the dark, in 1 mL of KRPG buffer (145 mM NaCl; 5.7 mM K_2HPO_4; 4.86 mM KCl; 0.54 mM $CaCl_2$; 1.22 mM $MgSO_4$; 5.5 mM glucose; pH 7.35) with 10 μM Amplex Red® and 0.2 U/mL of Horse Radish Peroxidase (HRP) per 100 mg of fresh weight. Catalase, an H_2O_2 scavenger, was used as a control. After 10 min of incubation at 4°C with catalase (1 unit/μL), 10 μM Amplex Red® and 0.2 U/mL of HRP were added to the samples. After centrifugation (10,000×*g*, 15 min, 4°C), 100 μL of supernatant were used to quantify resorufin ($\lambda_{ex} = 560$ nm; $\lambda_{em} = 584$ nm) by spectrofluorimetry (Mithras, Berthold Technology). The relative fluorescence units were converted into μmol of H_2O_2 mg^{-1} root fresh weight on the basis of a standard curve established from known concentrations of H_2O_2.

Nitric Oxide and Peroxynitrite Quantification

$ONOO^-$ and NO concentrations were determined using A17 or transformed roots ground on ice and in the dark, with 1 mL of Tris-HCl (10 mM, pH 7.5), KCl (10 mM) buffer

with 5 μM aminophenyl fluorescein (APF) or 10 μM 4,5-diaminofluorescein (DAF), respectively, per 100 mg of fresh weight. Epicatechin, an $ONOO^-$ scavenger, was used as a control. After 10 min of incubation at 4°C with epicatechin (1 mM), APF was added to the samples at a final concentration of 5 μM. cPTIO, an NO scavenger, was used as a control. After 10 min of incubation at 4°C with cPTIO (500 μM), DAF was added to the samples at a final concentration of 10 μM.

After centrifugation (10,000×*g*, 15 min, 4°C), 100 μL of supernatant were used to quantify $ONOO^-$ or NO ($\lambda_{ex} = 485$ nm; $\lambda_{em} = 535$ nm) by spectrofluorimetry (Mithras, Berthold Technology).

S-nitrosothiol Quantification

S-nitrosothiol quantification was performed using the Saville–Griess assay (Gow et al., 2007). A17 roots or transformed roots were ground, on ice and in the dark, in extraction buffer (1 mL/100 mg of fresh weight, 0.1 M Tris-HCl, pH 7.5; 1 mM PMSF). After centrifugation (10,000×g, 15 min, 4°C), 100 μL of supernatant were incubated with 100 μL of buffer *A* (0.5 M HCl; 1% sulfanilamide) or 100 μL of buffer *B* (0.5 M HCl; 1% sulfanilamide; 0.2% $HgCl_2$). After incubation (15 min at room temperature), 100 μL of Griess reagent[(0.5 M HCl; 0.02% N-(1-naphtyl)-ethylenediamine dihydrochloride] were added. After 15 min, SNOs were quantified by measuring absorbance at 540 nm. A standard curve was obtained using different concentrations of GSNO.

Nitrate Determination

Nitrate determination was performed according to Miranda et al. (2001), based on the reduction of nitrate to nitrite by vanadium and colorimetric detection at 540 nm of nitrite in the presence of sulfanilamide and N-1-naphthylethylenediamine. Approximately 100 mg of 7-day-old plant roots were collected, flash-frozen in liquid N_2, and ground into powder. Three hundred micro liter of ultra-pure water were added to 20 mg of frozen sample, thoroughly vortexed, and incubated with occasional mixing for 15 min on ice. After centrifugation 15 min at 13,000×*g* and 4°C, the supernatant was recovered and used for nitrate determination.

Nitrate Reductase Activity Measurements

Transformed root samples were frozen in liquid nitrogen and ground using pestle and mortar. Extraction was performed in MOPS buffer (1 mL per 100 mg of fresh weight, 50 mM MOPS-KOH buffer, pH 7.6; 5 mM NaF; 1 μM Na_2MoO_4; 10 μM FAD; 1 μM leupeptin, 0.2 g/g FW polyvinylpolypyrrolidone; 2 mM β-mercaptoethanol; 5 mM EDTA). After centrifugation (20,000×*g*, 5 min, 4°C), the supernatant was used to measure NR activity. The reaction mixture consisted of 50 mM MOPS-KOH buffer, pH 7.6, containing 1 mM NaF, 10 mM KNO_3, 0.17 mM NADH, and 5 mM EDTA. After incubation 15 min at 30°C, the reaction mixture was stopped by adding an equal volume of sulfanilamide (1% w/v in 3 N HCl) followed by N-naphtylethylenediamine dihydrochloride (0.02%, w/v), and the A_{540} was measured. A standard curve was obtained based on different concentrations of nitrite.

GSNOR Activity Measurements

To measure GSNOR activity, roots were ground in liquid nitrogen and proteins were extracted in 50 mM Tris-HCl buffer, pH 8, 0.5 mM EDTA, and 1 mg/mL of a protease inhibitor cocktail (1 mL of buffer per 100 mg of fresh weight). GSNOR activity was assayed from the rate of NADH oxidation by measuring the decrease in absorbance at 340 nm at 25°C using 25 μg of proteins in a total volume of 200 μL of extraction buffer containing 350 μM NADH with or without 350 μM GSNO. GSNO reductase activity was determined by subtracting NADH oxidation values in the absence of GSNO from values in the presence of GSNO. All samples were protected from light during the assay and tested for linearity. A standard curve was obtained using different concentrations of NADH.

Statistical Analyses

Statistical analyses were performed using one- or two-way analysis of variance (ANOVA) followed by Fisher's test. Data were considered as significantly different when $p < 0.05$.

RESULTS AND DISCUSSION

NO Homeostasis Participates in the *M. truncatula* Immune Response

To investigate the putative role of NO homeostasis in the *M. truncatula*/*A. euteiches* interaction, roots were transformed to inactivate the NR-encoding *MtNIA1/2* genes or to overexpress GSNOR-encoding genes. Quantification of gene transcripts in transformed roots using RT-qPCR confirmed that the two *NIA* genes were repressed (**Figure 1A**) while *GSNOR* was overexpressed (**Figure 1B**). To perform functional validation of the different constructs, we quantified NO and SNO levels in transformed roots. The two genetic manipulations modulated NO or SNO levels (**Figure 1**). SNO levels remained unchanged in *RNAi::MtNIA1/2* roots as compared to the controls, whereas NO levels clearly decreased (**Figure 1A**). This was in accordance with the downregulation of NR, a major enzymatic source of NO. Conversely, NO levels in the *35S::GSNOR* roots did not significantly change, but SNO significantly increased as compared to control roots (**Figure 1B**). This was surprising because in most previous experiments a negative correlation was described between SNO levels and GSNOR activity (Feechan et al., 2005; Rusterucci et al., 2007; Yun et al., 2011). However, it is interesting to note that in pea (a legume closely related to *M. truncatula*), higher SNO levels induced by wounding were correlated with higher GSNOR activity (Corpas et al., 2008).

We studied the impact of these genetic transformations on the *M. truncatula*/*A. euteiches* interaction. ELISA tests using antibodies raised against *A. euteiches* (Slezack et al., 1999) were performed to quantify the presence of the pathogen in roots. In *RNAi::MtNIA1/2* roots (**Figure 2A**), *A. euteiches* colonization was significantly greater than in control transformed roots

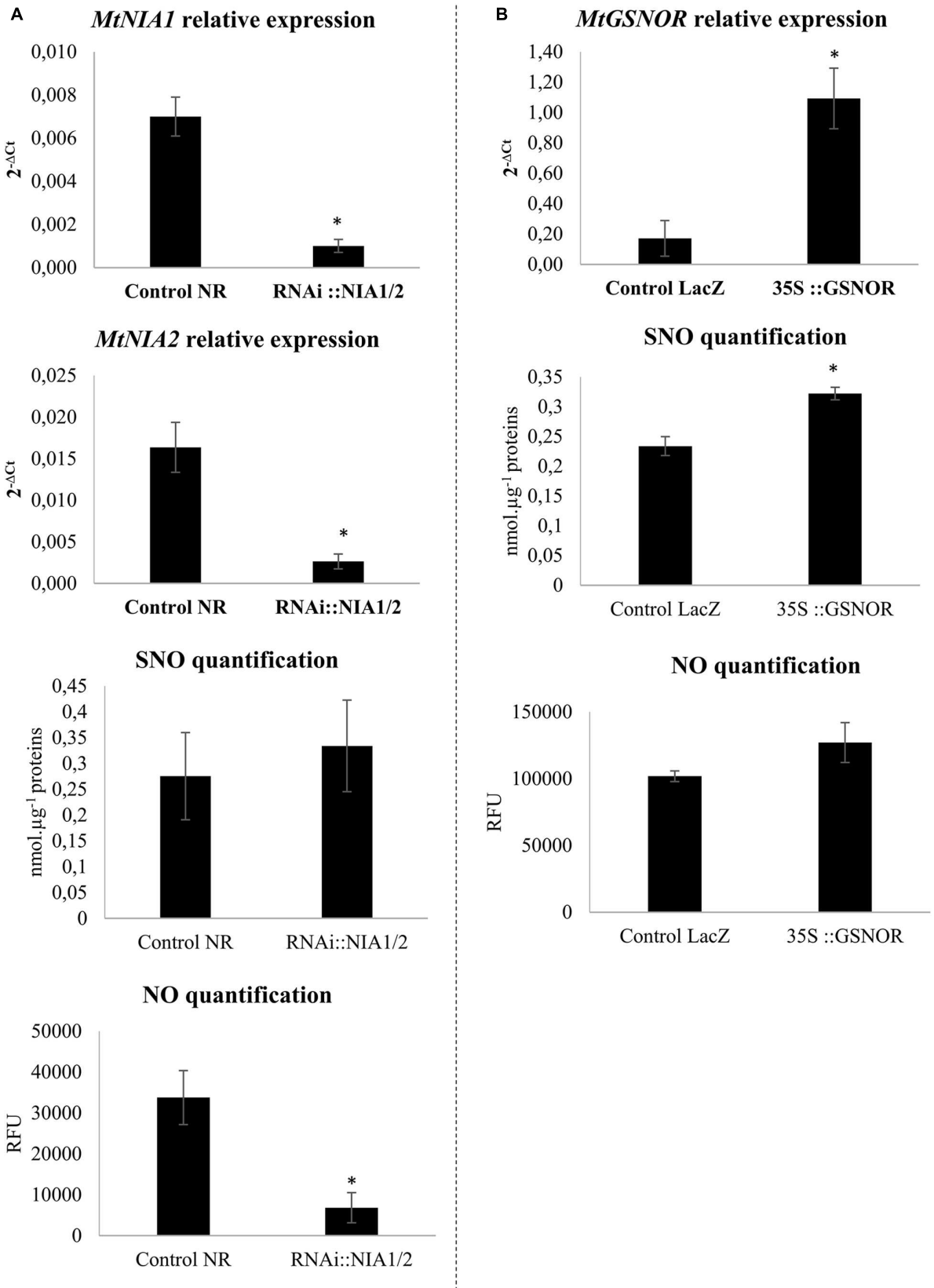

FIGURE 1 | Continued

FIGURE 1 | Continued

Transformed root validation. **(A)** Transcript levels of *MtNIA1* and *MtNIA2* in *RNAi::NIA1/2*-transformed roots were compared to control transformed roots (control NR). SNO quantification using the Saville–Griess assay and NO quantification using the fluorophore DAF (10 μM). Control NR and *RNAi::NIA1/2*-transformed roots extracts were pre-incubated or not with 500 μM cPTIO as an NO scavenger. **(B)** Transcript levels of *MtGSNOR* in *35S::GSNOR*-transformed roots were compared to control transformed roots (control LacZ). SNO quantification using the Saville–Griess assay and NO quantification using the fluorophore DAF (10 μM). Control LacZ and *35S::GSNOR*-transformed roots extracts were pre-incubated or not with 500 μM cPTIO as an NO scavenger. Error bars indicate standard errors ($n = 4$ for transcripts and NO levels; $n = 8$ for SNO levels), and * indicates significant differences ($p < 0.05$).

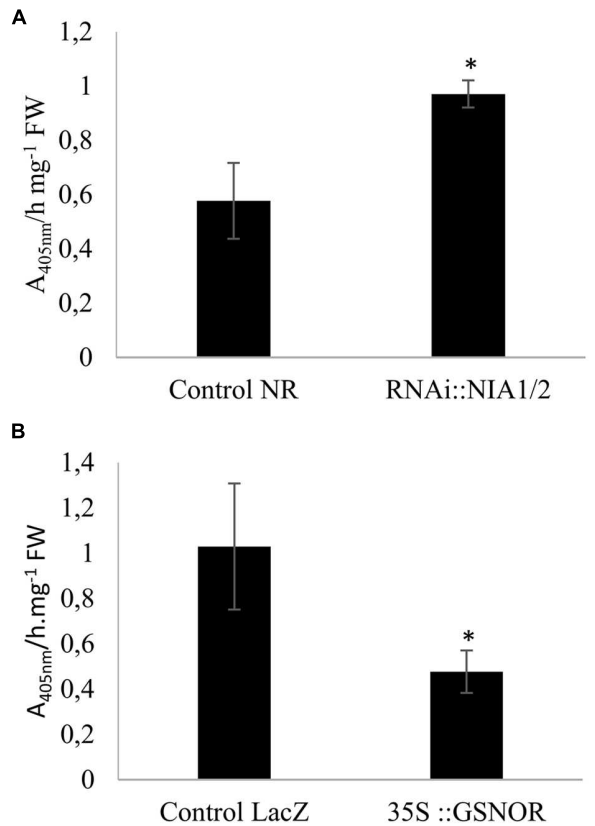

FIGURE 2 | Quantification of *Aphanomyces euteiches* in extracts from inoculated transformed roots. *RNAi::NIA1/2*-transformed roots **(A)** and *35S::GSNOR*-transformed roots **(B)** were extracted for ELISA tests. Roots were cultivated *in vitro* for 7 days on Fahraeus medium and then inoculated with *A. euteiches*. The background signal in non-inoculated roots was subtracted from the signal detected in inoculated roots. Error bars indicate standard errors ($n = 4$), and * indicates significant differences ($p < 0.05$). Data from one representative experiment out of four independent experiments.

(Control NR roots). These data reaffirm the role of the NR enzyme in the plant immune response. In *A. thaliana,* the NR-deficient double mutant (*nia1 nia2*) failed to exhibit a hypersensitive response and was hyper-susceptible to *P. syringae* (Modolo et al., 2006; Oliveira et al., 2009) and to the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* or *Botrytis cinerea* (Perchepied et al., 2010; Rasul et al., 2012). Although these effects were attributed to the substantially reduced NO levels in this mutant, a side effect of N metabolism on plant defense cannot be excluded as NR stands at the crossroads between N metabolism and NO production.

Our results using *GSNOR*-transformed roots showed that pathogen levels were lower in *GSNOR*-overexpressing roots (**Figure 2B**) than in control transformed roots (Control *LacZ* roots). GSNOR could therefore be considered as a positive regulator of *M. truncatula* resistance to *A. euteiches*. Previous works already investigated the physiological roles of GSNOR in plant-pathogen interactions, using transgenic *A. thaliana* plants (Feechan et al., 2005; Rusterucci et al., 2007; Yun et al., 2011). Results are sometimes contradictory, as modulation of *AtGSNOR* expression enhanced or decreased plant disease resistance depending on the pathosystem. GSNOR could play a significant role in plant immunity because GSNO is considered as a mobile reservoir of NO, is more stable than NO, and is a transnitrosylation agent of proteins. The contrasted results obtained in our study with *NR* and *GSNOR* constructs could be attributed to the specific roles of the corresponding proteins in NO homeostasis. NR is involved in NO synthesis, whereas the primary role of GSNOR is to regulate GSNO contents. The recent results from Yun et al. (2016) confirm that GSNO and NO may play distinct roles in plant immunity by acting on different molecular targets. In addition, GSNOR indirectly affects NO, GSH, ROS, and total intracellular nitrosothiol (SNO) levels, indicating that GSNOR might be more globally involved in the regulation of the cell redox state (Espunya et al., 2006; Yun et al., 2011).

Nitric Oxide partly regulates N metabolism. Therefore we also investigated the effects of *GSNOR* overexpression on root NO_3^- contents and NR activity in transformed roots. *GSNOR* overexpression increased basal NO_3^- content and NR activity (**Figures 3A,B**). Modulation of N metabolism by GSNO and NO in *A. thaliana* has been described (Frungillo et al., 2014), and was explained by the effect of NO and GSNO on NR activity and on the expression of the *AtNRT2.1* high-affinity NO_3^- transporter gene. Similarly to our data, that study shows that *GSNOR* overexpression is correlated with higher NR activity and NO_3^- content. Interestingly, we noted that pathogen colonization reduced NO_3^- concentrations in roots by approximately 65%, suggesting an effect of *A. euteiches* on nitrate transport and/or NO_3^- assimilation. Although we found a higher NO_3^- content in 35S::GSNOR-infected roots than in control infected roots, the amplitude of the pathogen-induced decrease in NO_3^- level was not impacted in *35S::GSNOR* roots, suggesting that this process is independent of GSNO homeostasis. This reduced level of NO_3^- is unlikely to result from consumption of NO_3^- by the pathogen: data mining of the *A. euteiches* database revealed that no homologs of the NR, NIR, and NO_3^- transporter (NRT2) genes were detected in the genome of this pathogen[2], confirming

[2]http://www.polebio.lrsv.ups-tlse.fr/aphano/

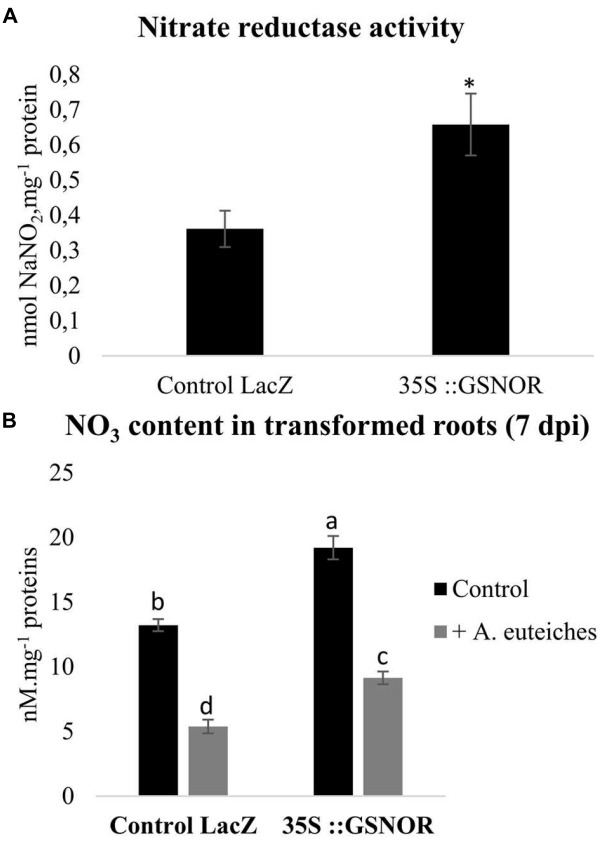

FIGURE 3 | Nitrate reductase (NR) activity and NO_3^- contents in transformed roots. (A) NR activity in control transformed roots (Control LacZ roots transformed with pK7GWG2D-GFP) and in transformed roots overexpressing GSNOR (*35S::GSNOR*). Transformed roots were cultivated *in vitro* on Shb10 medium. **(B)** NO_3^- concentrations in control transformed roots (Control LacZ) and *GSNOR*-overexpressing roots. Transformed roots were cultivated *in vitro* for 7 days on Fahraeus medium, and inoculated with *A. euteiches*. Error bars indicate standard errors ($n = 4$), and letters or * indicate significant differences ($p < 0.05$). Data from one representative experiment out of three independent experiments for both NR activity and NO_3^- contents ($n = 12$).

earlier observations that NO_3^- is unfavorable for *A. euteiches* development (Huber and Watson, 1974). Alternatively, we cannot exclude that the decreased NO_3^- content in infected roots could be due to nitrate leakage from the roots related to developing necrosis.

Effect of N Nutrition on NO/H_2O_2/$ONOO^-$ Accumulation and SNO Contents

To analyze the role of N availability on NO, H_2O_2, and $ONOO^-$ accumulation, *M. truncatula* plants were cultivated in complete medium or NO_3^--deficient medium (NØ), and inoculated or not with *A. euteiches*. The NO scavenger cPTIO and the $ONOO^-$ scavenger epicatechin were used as controls to check the specificity of the fluorescence probes. We observed that NO_3^- deficiency caused a significant increase in $ONOO^-$ content on NØ medium (**Figure 4C**), whereas NO and H_2O_2 levels decreased (**Figures 4A,B**), highlighting a link between NO_3^- content and production of these reactive species. A clear effect of pathogen colonization was only evidenced for H_2O_2 contents (**Figure 4A**), and this increase was abolished on NØ. Surprisingly, although NO production is considered as a common response to pathogens, no increase in NO levels was detected in response to *A. euteiches* (**Figure 4B**). More generally, whereas NO, ROS, or $ONOO^-$ production has been widely described in response to pathogens, the literature does not give a clear picture of the cross-talks between these molecules. For instance, we observed a negative correlation between NO and $ONOO^-$ contents in response to NO_3^- deficiency, but in other models high NO levels are often correlated with high $ONOO^-$ levels (Abramowski et al., 2015; Kulik et al., 2015). These conflicting observations raise some questions. Are these discrepancies due to plant models or due to the difficulty in measuring and precisely localizing these molecules? Differences in the stability of these molecules or their specific scavenging by plants during pathogen attack could explain why we did not detect changes in $ONOO^-$ or NO levels in response to *A. euteiches*. Moreover, NO could also be used by the pathogen to activate its own metabolism, an important step in plant infection by fungi (Sedlářová et al., 2016).

We also measured root SNO levels and GSNOR activity in the biological conditions of interest. Root SNO contents, determined using the Saville–Griess method, significantly increased on NØ medium as compared to the complete medium (**Figure 4D**). In response to *A. euteiches,* no significant change in SNO levels was highlighted (**Figure 4D**). Therefore, on NØ medium, the SNO content evolved in an opposite way to the NO content, similarly to the $ONOO^-$ content. This result is in accordance with results reported in *Helianthus annuus* (Chaki et al., 2011), and can be attributed to the fact that NO is the source for $ONOO^-$ and SNO. By contrast, a high NO content can be correlated with a high SNO content when plants are grown on culture medium containing NO_3^- (Abramowski et al., 2015; Pietrowska et al., 2015). Our data also suggest that NO_3^- nutrition impacts the overall balance between NO, $ONOO^-$, and SNO. Regarding GSNOR, no changes in its activity was detected upon inoculation, in line with the absence of change in SNO levels in infected roots. In the roots of plants cultivated on NØ (**Figure 5**), higher GSNOR activity was correlated with higher SNO levels, confirming the positive correlation between GSNOR activity and SNO levels observed in 35S::GSNOR-transformed *Medicago* roots (**Figure 1**) and in pea, a closely related legume (Corpas et al., 2008). The positive or negative correlation between GSNOR activity and SNO levels or between NO and SNO levels depending on plant species and experimental conditions can be explained by several hypotheses. The SNO level is regulated through nitrosylation and denitrosylation; GSNOR, by controlling the level of GSNO, indirectly affects the level of *S*-nitrosylation. However, the TRX (thioredoxin)/NTR (NADPH-dependent TRX reductase) enzymatic system also controls *S*-nitrosylation (Kneeshaw et al., 2014). Interestingly, these activities were also identified in roots and activated by NO, leading to denitrosylation of specific proteins (Correa-Aragunde et al., 2015). Thus, these results, together with our study, illustrate the complex relationships

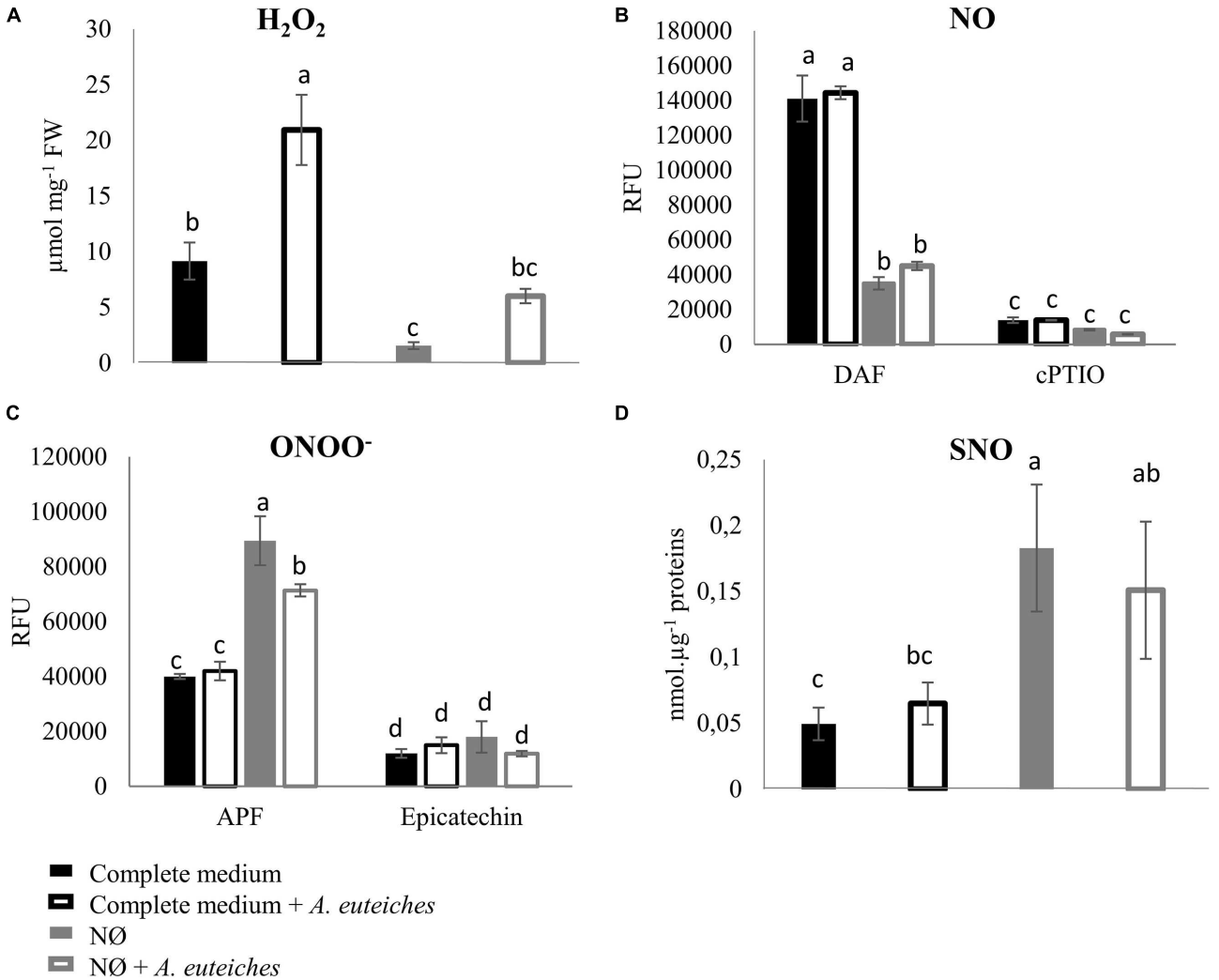

FIGURE 4 | H_2O_2, NO, $ONOO^-$, and SNO quantification in *Medicago truncatula* roots 7 dpi. *M. truncatula* was cultivated on complete medium or NØ medium, and roots were harvested 7 days after inoculation with *A. euteiches* and used to detect H_2O_2, NO, and $ONOO^-$ concentrations using fluorescent probes, and SNO concentrations using the Saville–Griess assay. **(A)** H_2O_2 quantification using 10 µM Amplex Red® fluorophore and 0.2 U/mL of peroxidase. Catalase (1 U/µL), used as an H_2O_2 scavenger, abolished Amplex Red® fluorescence. **(B)** NO quantification using the fluorophore DAF (10 µM). Root extracts were pre-incubated or not with 500 µM cPTIO as an NO scavenger. **(C)** $ONOO^-$ quantification using the fluorophore APF (5 µM). Root extracts were pre-incubated or not with 1 mM of the $ONOO^-$ scavenger epicatechin. **(D)** SNO quantification by the Saville–Griess assay. Error bars indicate standard errors ($n = 4$ for **A–C**; $n = 14$ for **D**), and letters indicate significant differences ($p < 0.05$). Data from one representative experiment out of three independent experiments for H_2O_2, NO, and $ONOO^-$ concentrations, and data corresponding to two independent experiments pooled together for SNO concentrations. RFU, relative fluorescence units.

between NO production/GSNOR activity and total SNO levels. Abiotic stresses also increase GSNOR activity (Kubienova et al., 2014), and this appears to be the case for *M. truncatula* plants under NO_3^- deficiency. Higher GSNOR activity in N-deprived roots (**Figure 5**) could lead to a physiological state inducing higher resistance to *A. euteiches,* as observed in the 35S::GSNOR-transformed roots (**Figure 2B**). This could partly explain the enhanced resistance to this oomycete on NØ medium despite the low levels of NO in the roots. Thus, altogether our data highlight the possible positive and non-redundant roles of NO (**Figures 1A** and **2A**) and SNO (**Figures 1B**, **2B**, and **4D**) in mediating *M. truncatula* resistance to *A. euteiches*.

CONCLUSION AND NEW HYPOTHESES

The results obtained in the present study are summarized in **Figure 6**. We have demonstrated, using transformed roots affected in genes involved in NO synthesis (*NIA* genes) and turnover (GSNOR gene), that deregulation of NO homeostasis has an effect on *M. truncatula* resistance against *A. euteiches,* as observed in other pathosystems (1). In addition, it appears that the modulation of NO homeostasis (through GSNOR overexpression) impacts NR activity and NO_3^-content, indicating possibly an effect of GSNOR (or GSNO) on basal NO_3^- transport/assimilation

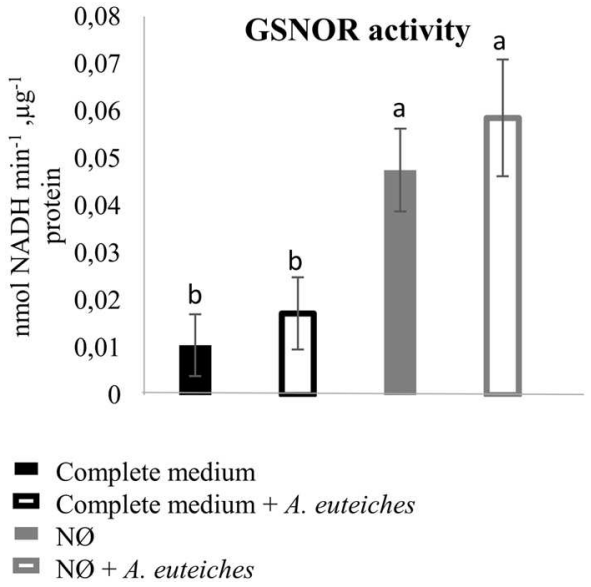

FIGURE 5 | GSNO reductase activity in *M. truncatula* roots 7 dpi. *M. truncatula* seedlings were inoculated or not with *A. euteiches*, and cultivated on complete medium or NØ medium for 7 days. Root extracts were used to measure GSNOR activity. Error bars indicate standard errors ($n = 4$), and letters indicate significant differences ($p < 0.05$). Data from one representative experiment out of four independent experiments.

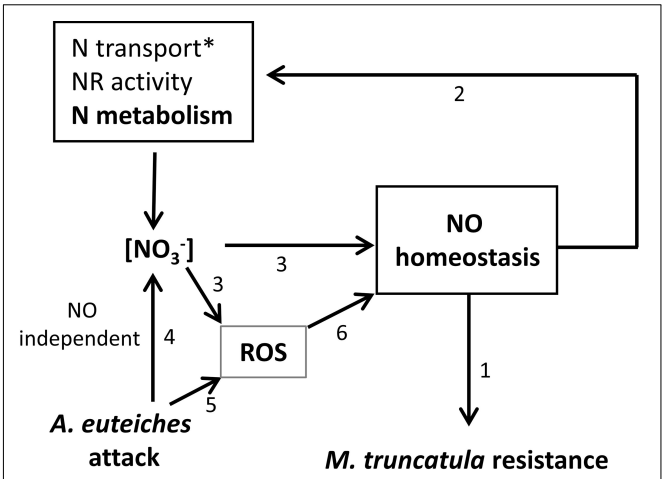

FIGURE 6 | Working model. Results from the present work indicate that *RNAi::MtNIA1/2* and *35S::GSNOR* transformed roots are, respectively, more susceptible and more resistant to *A. euteiches* (1). NR activity and NO_3^- content were impacted by GSNOR overexpression, indicating a possible effect of GSNOR on basal NO_3^- transport/assimilation (2). NO_3^- availability in the medium causes quantitative modulation of ROS/RNS/NO content and affects their balance (3). Infection by *A. euteiches* decreases root NO_3^- content (4) and induces higher ROS levels (5). According to the literature superoxyde ($O_2^{\cdot-}$), by reacting with NO to form peroxynitrite, can influence the concentration of NO available for signaling (6). *: GSNO was shown to regulate NO_3^- uptake through transcriptional regulation of NRT2.1 (Frungillo et al., 2014).

and confirming the results of Frungillo et al. (2014) (2). In return, NO_3^- availability in the medium can affect NO homeostasis by modulating ROS/RNS/NO contents and their balance (3). Finally, infection by *A. euteiches* decreases root NO_3^- content (4) and induces higher ROS levels (5). Altogether these results highlight the close interplay occurring between N nutrition and NO homeostasis as well as the involvement of NO in the modulation of plant resistance by N nutrition.

Future work should take into account the role of N availability on NO-mediated plant molecular responses. Thus, the study of the specific role of GSNO in this process through the identification *S*-nitrosylated/denitrosylated proteins under different N availability conditions and N sources seems promising. A focus will be made on proteins involved in the plant immune response (1), but also on the feedback regulation of N metabolism by NO because NO could control NO_3^- availability and therefore plant resistance (2) (**Figure 6**). Investigations using foliar pathogens and other plant models will lead to a possible generalization of this phenomenon. More generally, plant N use efficiency can be affected by NO since NO controls not only N metabolism but also plant root growth and architecture changes in response to NO_3^- (Manoli et al., 2014; Sun et al., 2015). Recent data show that plant N use efficiency and N-induced susceptibility to pathogens may be linked (Ballini et al., 2013). Consequently future studies should also focus on candidate proteins involved in root development. Finally, experiments conducted with plant genotypes differing in their resistance levels will permit to study the quantitative effect of NO/ROS production on plant defense.

AUTHOR CONTRIBUTIONS

ET, H-NT, ABo, and SJ conceived and designed the research; ET, H-NT, CF, ABo, and ABe carried out the experiments and analysis/interpretation of data; ET, H-NT, DW, and SJ wrote the manuscript. All authors contributed to the discussion and approved the final manuscript.

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Systemic Induction of NO-, Redox-, and cGMP Signaling in the Pumpkin Extrafascicular Phloem upon Local Leaf Wounding

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Cucurbits developed the unique extrafascicular phloem (EFP) as a defensive structure against herbivorous animals. Mechanical leaf injury was previously shown to induce a systemic wound response in the EFP of pumpkin (*Cucurbita maxima*). Here, we demonstrate that the phloem antioxidant system and protein modifications by NO are strongly regulated during this process. Activities of the central antioxidant enzymes dehydroascorbate reductase, glutathione reductase and ascorbate reductase were rapidly down-regulated at 30 min with a second minimum at 24 h after wounding. As a consequence levels of total ascorbate and glutathione also decreased with similar bi-phasic kinetics. These results hint toward a wound-induced shift in the redox status of the EFP. Nitric oxide (NO) is another important player in stress-induced redox signaling in plants. Therefore, we analyzed NO-dependent protein modifications in the EFP. Six to forty eight hours after leaf damage total S-nitrosothiol content and protein S-nitrosylation were clearly reduced, which was contrasted by a pronounced increase in protein tyrosine nitration. Collectively, these findings suggest that NO-dependent S-nitrosylation turned into peroxynitrite-mediated protein nitration upon a stress-induced redox shift probably involving the accumulation of reactive oxygen species within the EFP. Using the biotin switch assay and anti-nitrotyrosine antibodies we identified 9 candidate S-nitrosylated and 6 candidate tyrosine-nitrated phloem proteins. The wound-responsive Phloem Protein 16-1 (PP16-1) and Cyclophilin 18 (CYP18) as well as the 26.5 kD isoform of Phloem Protein 2 (PP2) were amenable to both NO modifications and could represent important redox-sensors within the cucurbit EFP. We also found that leaf injury triggered the systemic accumulation of cyclic guanosine monophosphate (cGMP) in the EFP and discuss the possible function of this second messenger in systemic NO and redox signaling within the EFP.

Keywords: phloem, systemic, wound response, signaling, NO, redox, antioxidant system, cGMP

INTRODUCTION

Leaf damage by insects, herbivores or abiotic sources triggers wound responses in plants (Savatin et al., 2014). Affected tissues rapidly cumulate defensive proteins, protease inhibitors and secondary metabolites for fighting off herbivorous attackers or microbial invaders. Subsequently, the wound site is sealed by induction of localized cell death and callose formation. These responses are concerted amongst others by the general stress messengers hydrogen peroxide (H_2O_2), nitric oxide (NO) and the more damage-specific phytohormone jasmonic acid (JA) (Orozco-Cárdenas et al., 2001; Wünsche et al., 2011; Zebelo and Maffei, 2015). Interactions of NO and H_2O_2 with calcium and cyclic guanosine monophosphate (cGMP) have been frequently reported although little is known about a role of cGMP in wound signaling (Lin et al., 2011, 2012).

Plants do not only react locally to a threat but signals are often translocated to distant plant parts which are thus prepared for the upcoming stress encounter. Several current reports suggested functions of calcium, calcium-dependent electric signals, H_2O_2-autopropagation waves, NO and JA in the plant systemic wound response (SWR) (Mousavi et al., 2013; Gilroy et al., 2014; Zebelo and Maffei, 2015). However, the exact sequence and mode of interaction between these signals remains to be deciphered, and transport routes are also not well defined for all signals. The phloem distributes assimilates from autotrophic to heterotrophic plant parts and was shown to be involved in signal transport during SWR. For instance, JA and NO derivatives were detected within the vasculature after leaf wounding (Glauser et al., 2009; Chaki et al., 2011; Espunya et al., 2012; Furch et al., 2014) while electrical signals spread in a pattern, which was at least to some extent dependent on phloem connectivity but independent of assimilate flow (Salvador-Recatalà et al., 2014). Micromolar concentrations of H_2O_2 induced within seconds calcium-dependent NO production in the phloem of *Vicia faba* (Gaupels et al., 2008). Taken together these findings argue for complex defense signaling within the phloem.

Due to its high nutrient content the phloem is an attractive target for insect and pathogen attack and therefore disposes of effective defense mechanisms. Cucurbits have developed the unique extrafascicular phloem (EFP), which forms a specialized defense structure against herbivores similar to laticifers in other plant species (Konno, 2011; Gaupels and Ghirardo, 2013). Exudates from the EFP contain toxic cucurbitacin steroids, alkaloids and terpenoids as a preformed barrier against invaders (Konno, 2011). Moreover, metabolomic and proteomic approaches revealed that leaf damage triggered SWR in the EFP of *Cucurbita maxima* (pumpkin) amongst others by JA and redox signaling (Gaupels et al., 2012).

In the present study, we aimed at further exploring signal transduction induced in the EFP during systemic wound responses. We were particularly interested in alterations of the antioxidant system as a hint toward induced redox changes and in signaling by NO-mediated protein modifications and cGMP. The observed damage responses might be transmitted over long distances via the phloem or could be part of EFP-internal defense mechanisms triggered by systemic messengers.

MATERIALS AND METHODS

Plant Treatment and Sampling

Leaf edges of 4–5 week-old pumpkin plants (*Cucurbita maxima* cv. Gele Centenaar) grown under green-house conditions were crushed between the lids of two 50 ml polypropylene reaction tubes. Control plants were left untreated. Phloem sap was collected as described earlier (Gaupels et al., 2012). Petioles and stems were cut using a razor blade and the basal side of the cut was immediately blotted with tissue paper. The exuding phloem sap was subsequently collected by a micropipette and mixed with an equal volume of phloem buffer (50 mMTris/HCl, pH 7.8, 0.1% β-mercaptoethanol; McEuen and Hill, 1982). Pumpkin leaf extracts were prepared by grinding 0.5 g leaf material in liquid N_2, addition of 3 ml homogenization buffer (50 mM TrisCl, pH 7.8, 1 mM EDTA, 7.5% [w/v] soluble polyvinylpyrrolidone, 2 mM ascorbate) and subsequent centrifugation. The supernatant was used for APX measurements.

Measurements of Antioxidant Enzymes, Glutathione and Ascorbate

All enzyme measurements were done with an Ultrospec 3100 Pro photometer (GE Healthcare Life Sciences) following previously published protocols (Harrach et al., 2008). APX activity was measured in 36 µl phloem sample (phloem exudate plus phloem buffer) or 50 µl leaf extract while 10 and 32 µl aliquots of phloem samples were used for determination of DHAR and GR activities, respectively. For the glutathione and ascorbate measurements 10 µl phloem exudate was added to 90 µl of 5% meta-phosphoric acid. Samples were incubated for 10 min at RT and centrifuged for 30 min at 14000 rpm. The supernatant was stored at −20°C until further analysis. Immediately before the measurements samples were neutralized by adding 25 µl 1 M triethanolamine. Glutathione was measured in 5 µl neutralized extract using the Amplite™ Fluorimetric Glutathione GSH/GSSG Ratio Assay Kit (AAT Bioquest) following the manufacturer's instructions.

For ascorbate measurements, a colorimetric protocol was used (Harrach et al., 2008). Five microliter neutralized phloem extract was mixed with 150 µl 150 mM $NaPO_4$ (pH 7.4) and 150 µl H_2O_{dest} to determine reduced ascorbate. For the measurement of total ascorbate neutralized extract was mixed with 150 µl 150 mM $NaPO_4$ and 75 µl 10 mM dithiothreitol. After 10 min incubation at RT 75 µl 0.5% N-ethylmaleimide was added to the sample. The reaction protocol is the same for both reduced and total ascorbate. The sample was combined with 300 µl 10% (w/v) trichloroacetic acid, 300 µl 44% (v/v) phosphoric acid, 300 µl 4% (w/v) bipyridyl (in 70% EtOH), and 150 µl 2% (w/v) $FeCl_3$. After 1 h incubation at 37°C the absorption of the sample was measured at 525 nm.

Thiobarbituric Acid Reactive Substances Determination

Proteins were removed from 100 µl phloem exudate by adding 200 µl ice-cold trichloroacetate, incubation for 15 min on ice and subsequent centrifugation. Two hundred fifty microliters of the supernatant was used for determining the content of

thiobarbituric acid reactive substances (TBARS) according to Hodges et al. (1999).

Determination of the Total S-nitrosothiol Content

The total S-nitrosothiol content of phloem sap was analyzed by a Nitric Oxide Analyzer (Siever's NOA 280i, GE Power and Water, Analytix). Seventy five microliters phloem exudate was treated for 10 min at RT with 19 µl 5% sulfanilamide (w/v, in 1 M HCl) in order to scavenge nitrite. The sample was then injected into the NOA reaction vessel which contained a reducing triiodide solution (Piknova and Schechter, 2011). Released NO reacted with ozone generated by the NOA and the resulting chemiluminescence was recorded by an internal detector. Peak area integration and calculation of SNO concentrations based on nitrite standards were performed using the Siever's NO Analysis Software.

Investigation of Protein S-nitrosylation by the Biotin Switch Assay

S-nitrosylation of protein Cys residues was investigated by the biotin switch method (Jaffrey and Snyder, 2001). This approach is based on blocking free Cys residues by methanethiosulfonate (MMTS) or iodoacetamide, breaking of NO-Cys bonds by ascorbate and subsequent attachment of biotin to formerly S-nitrosylated Cys. Biotinylated proteins can then be detected in WB analysis or isolated using streptavidin-coated magnetic beads. For WB analyses phloem proteins were irreversibly alkylated by iodoacetamide which prevented sample gelation through redox-dependent polymerization of PP1 and PP2. For this, 1 volume of EFP exudate was mixed with 2 volumes of 200 mM iodoacetamide in HEN (25 mM HEPES/NaOH, 1 mM EDTA, 0.1 mM neocuproin, pH 7.7)/1% sodium dodecyl sulfate (SDS) and then incubated for 30 min at room temperature. Proteins were precipitated with ice-cold acetone, re-dissolved in 1 volume (of the original phloem sample) 1 mM ascorbate/1 mM Biotin-HPDP (Pierce) and incubated for 1 h at RT for de-nitrosylation and subsequent biotinylation. After another acetone precipitation proteins were dissolved in non-reducing loading buffer, separated by SDS-PAGE, transferred to a nitrocellulose membrane by semi-dry blot and finally immuno-detected by an monoclonal anti-biotin antibody coupled to alkaline phosphatase (Sigma) using a commercial substrate.

PP1 and PP2 are highly abundant in phloem sap masking other phloem proteins in down-stream analyses. Therefore, it was necessary to remove these proteins before isolation and identification of S-nitrosylated phloem proteins by MS. Polymerization of the redox-sensitive PP1 and PP2 was induced by adding 400 µl alkaline HEN buffer to 100 µl phloem exudate (Alosi et al., 1988). The PP1 and PP2 containing gel-like matrix was removed by a pipet tip and the liquid phase was further analyzed. One hundred microliters sample was incubated for 45 min at RT with 400 µl 10 mM GSH for removing SNO groups or GSNO for *in vitro* S-nitrosylation of amenable Cys residues. Free Cys residues were blocked by adding 50 µl 25% SDS and 1.5 µl 10.6 M MMTS (in dimethylformamide) followed by 1 h incubation at 50°C with regular vortexing. Proteins were precipitated with 2 volumes ice-cold acetone. S-nitrosylated proteins were biotinylated by dissolving the precipitated proteins in 20 µl 1 mM ascorbate/1 mM Biotin-HPDP (Pierce) and incubation for 1 h at room temperature. After another acetone precipitation the resulting pellet was dissolved in 200 µl HEN/1% SDS and 400 µl neutralization buffer (20 mM HEPES/NaOH; 1 mM EDTA; 100 mM NaCl; 0.5% Triton X-100; pH 7.7).

We used streptavidin-coated magnetic beads (Dynabeads, Invitrogen) for isolation of biotinylated proteins according to the manufacturer's instructions. All washing steps were done with neutralization buffer. Biotinylated proteins were eluted by incubating the beads in 4 × Laemmli buffer for 5 min at 90°C. SDS-PAGE, colloidal coomassie staining, and the determination of partial amino acid sequences were performed as described in Walz et al. (2002). In short, gels were stained with colloidal Coomassie stain (Novex) over night and destained for 2 h in water. Protein bands were excised, destained, dehydrated, and digested overnight with modified trypsin (Roche Diagnostics). Peptides were extracted and vacuum-dried. Desalted digests were analyzed by an electrospray ionization quadrupole time-of-flight tandem mass spectrometer (Q-TOF, Micromass/Waters). After data processing and analysis of the resultant peptide fragmentation data, database similarity searches with the derived sequence stretches were performed with the short sequence blast algorithm (http://www.ncbi.nlm.nih.gov/blast/) against the non-redundant protein database, limited to green plants.

Investigation of Tyrosine Nitration

For WB analyses we loaded 0.5 µl sample in 50 µl phloem buffer on a nitrocellulose membrane using a 96-well vacuum dot-blot device (Bio-Dot, BioRad). Equal loading was checked by Ponceau Red staining of the blot membrane before blocking with 1% (w/v) milk powder. The primary antibody against nitrotyrosine (monoclonal anti-nitrotyrosine antibody, mouse, clone 1A6, Upstate) was diluted 1:1000 and the second antibody (anti-mouse-horse raddish peroxidase conjugate, goat, Invitrogen) was diluted 1:20000. After addition of the chemiluminescent peroxidase substrate, X-ray films were exposed to the blot membranes for 10–20 s and developed.

Tyrosine nitrated phloem proteins were immunoprecipitated using an anti-nitrotyrosine monoclonal antibody coupled to agarose (clone 1A6, Cayman). Fifty microliters phloem sample (exudate plus buffer) was diluted with 450 µl PBS containing 0.05% Tween-20. After adding 25 µl of antibody suspension the sample was incubated for 2 h at RT under agitation, centrifugated and 3 times washed with PBST. Finally, bound proteins were eluted by 5 min incubation at 95°C in 25 µl denaturating Laemmli buffer. SDS-PAGE and silver staining procedures were like previously described (Gaupels et al., 2012). For identification of tyrosine nitrated EFP proteins silver stained bands were manually cut, destained and washed with buffer containing 50 mM NH_4HCO_3 in 30% acetonitrile (ACN) and equilibrated in 10 mM NH_4HCO_3 prior to proteolytic digestion. Gel pieces were shrunk with 100% v/v ACN and rehydrated in 10 mM NH_4HCO_3. This treatment was repeated, followed by the addition of 0.1–0.2 µg of modified trypsin (SIGMA)

per piece. Digestion was carried out overnight at 37°C. The supernatant was collected and combined with the eluates of subsequent elution steps with 80% v/v ACN, 1% v/v TFA. The combined eluates were dried in a SpeedVac centrifuge. The dry samples were dissolved in 20 µl 50% v/v ACN, 0.1% v/v TFA for the subsequent MALDI preparation. Therefore, 0.5 µl of a 1:1 mixture of sample and a matrix solution consisting of 5 mg/mL CHCA (Bruker, Bremen, Germany) were spotted on a MALDI target.

Mass spectra were acquired using a Proteomics Analyzer 4700 (MALDI-TOF/TOF) mass spectrometer (Applied Biosystems, Framingham, USA). Measurements were performed with a 355 nm Nb:YAG laser in positive reflector mode with a 20 kV acceleration voltage. For each MS and MS/MS spectrum 3000 shots were accumulated. For each spot on a MALDI plate the eight most intense peptides were selected for additional MS/MS analysis. The acquired MS/MS spectra were searched with protein pilot software 3.0 against the Swiss-Prot database (updated August 2010; 519348 sequences, 183273162 residues) using an in-house version of Mascot 2.3.02. The resulting peptide fragmentation data were blast searched (http://www.ncbi.nlm.nih.gov/blast/) against the non-redundant protein database, limited to green plants.

cGMP Measurements

cGMP was extracted from 150 µl phloem sap by adding 300 µl EtOH followed by an incubation for 10 min at room temperature. After centrifugation the supernatant was vacuum-dried and stored at −20°C until further analysis. cGMP measurements by the cGMP Enzyme Immunoassay Kit (Sigma-Aldrich) were done according to the manufacturer's instructions. Reversed Phase chromatographic separation of 3′,5′-GMP was performed using a Shimadzu HPLC-system (Shimadzu). A Zorbax eclipse XCB-C18 1.8 µm column (50 × 4.6 mm; Agilent) connected to a C18-Security guard (Phenomenex) and a 2 µm column saver, kept at 25°C, was applied. The mobile phases were 3/97 methanol/water [v/v] (A) and 97/3 methanol/water [v/v] (B), each containing 50 mM ammonium acetate and 0.1% acetic acid. The following gradient was applied: 0–5 min, 0–50% B, and 5–8 min 0% B. The flow rate was 500 µL/min. Detection and quantification of cyclic nucleotides was carried out by a tandem mass spectrometer, 5500Q TRAP (AB Sciex), equipped with an electrospray ionization source, operating in positive ionization mode.

RESULTS AND DISCUSSION

The Antioxidant System in the EFP is Systemically Down-Regulated after Leaf Wounding

The EFP has a complete antioxidant system including various antioxidant enzymes and high levels of glutathione and ascorbate (Alosi et al., 1988; Walz et al., 2002; Lin et al., 2009). We focussed our work on ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) because they represent core elements of the Foyer-Halliwell-Asada cycle and displayed robust activities in phloem sap. All three investigated enzymes were significantly down-regulated already at 30 min after leaf squeezing (**Figures 1A–C**). Inhibition was most pronounced after 30 min for DHAR but much later at the 24 h time point for GR. APX underwent a bi-phasic depression of activity with minima at 0.5 and 24 h after wounding. Only an early drop in APX activity was observed in extracts from wounded leaf tissues (**Figure 1D**) suggesting that the bi-phasic inhibition of this enzyme was specific for the EFP.

The APX activity in phloem samples decreased by up to 54% when all leaves were wounded, whereas APX was down-regulated by 17% when just the two lowest leaves were wounded (**Figure 1E**). DHAR was not significantly affected (**Figure 1F**) whereas the GR activity was even increased by 28% at 3 h after the local stress treatment (**Figure 1G**). The results confirm that EFP responses to leaf damage are (1.) systemically transduced over long distances as well as (2.) dose- and/or distance-dependent in nature. It was previously observed that APX and catalase activities were transiently down-regulated in total leaf extracts of *Pelargonium peltatum* already at 5 and 8 h after leaf wounding, which caused an accumulation of H_2O_2 (Arasimowicz et al., 2009). Decreasing the antioxidant capacity of damaged and adjacent tissues is probably a mechanism for modulating defense signaling and wound healing by H_2O_2. The kinetics of APX inhibition are remarkably similar between pelargonium leaves, pumpkin leaves, and pumpkin EFP suggesting that down-regulation of antioxidant enzymes is part of a general plant response to acute stress.

A long-lasting reduction in antioxidant enzyme activities in the EFP would likely cause accumulation of reactive oxygen species (ROS) from different sources including energy metabolism and ROS generating enzymes. Under these conditions reduced glutathione (GSH) and ascorbate (AsA) would be oxidized to give glutathione disulfide (GSSG) and dehydroascorbate (DHA). Although both GSH as well as AsA levels were diminished after wounding there was no obvious accumulation of GSSG and DHA (**Figures 2A,B**). GSSG was even decreased whereas DHA levels were not significantly changed. The AsA/DHA ratio, which is a measure for the redox potential of the ascorbate pool, was lower at 0.5–3 h and 24 h after wounding whereas the GSH/GSSG ratio was even increased at these time points. Thus, rather than a consistent shift in GSH/GSSG and AsA/DHA ratios we observed a decline in total glutathione and ascorbate levels. Twenty four and 27% of free ascorbate but 23 and 32% of free glutathione disappeared from the EFP at 1 and 24 h after leaf damage. The wound-induced decrease in total ascorbate content is a common phenomenon reported for various plants (Suza et al., 2010). The bi-phasic drop in antioxidants matched well with the down-regulated activities of DHAR, GR and APX at these time points. The fate of antioxidants under severe stress conditions is not fully resolved but it is known that inhibition of GR and DHAR prevents recycling of AsA and GSH from DHA and GSSG. DHA is rather unstable and rapidly decays amongst others to oxalate and tartrate while GSSG binds to oxidized proteins by S-glutathionylation (Noctor and Foyer, 1998).

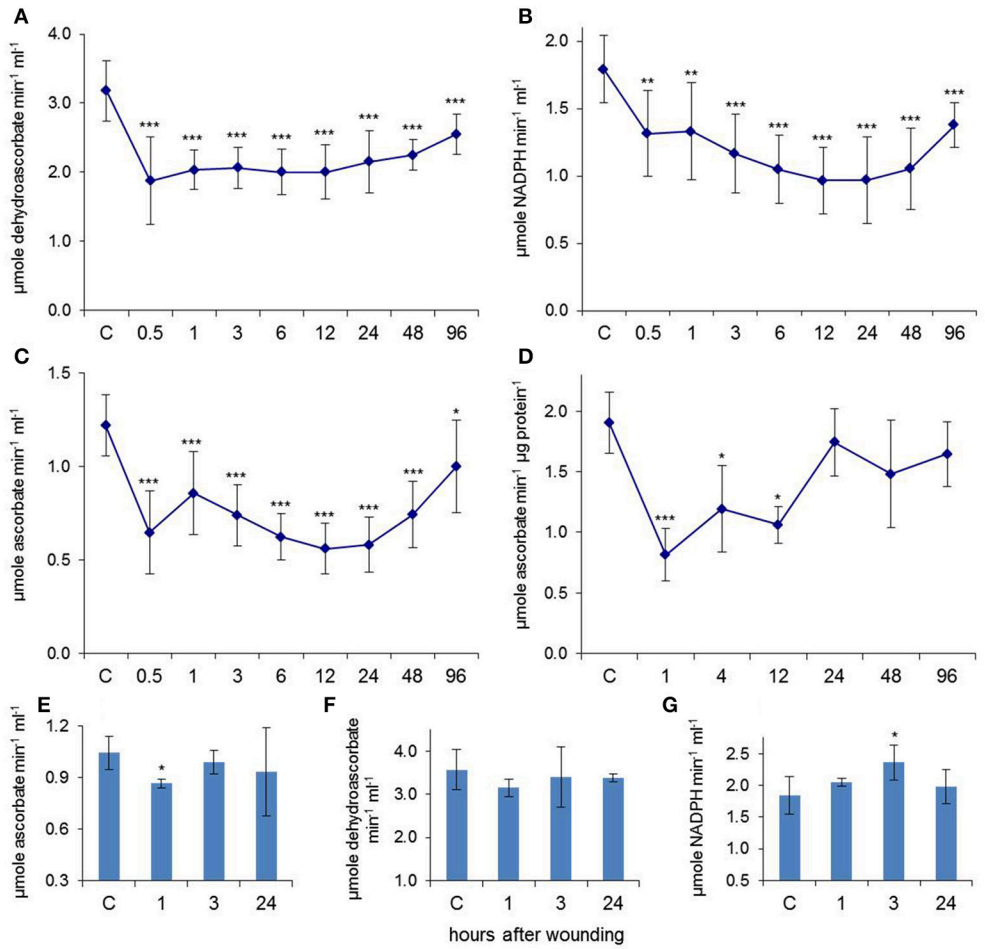

FIGURE 1 | Leaf wounding induced systemic regulation of antioxidant enzymes in the EFP. Enzyme activities of **(A)** DHAR, **(B)** GR, and **(C)** APX decreased in phloem exudates after wounding of all leaves. Data points represent means (±SD, $n = 9$–12). **(D)** APX activity was also reduced in extracts from injured leaf tissues ($n = 5$). **(E–G)** Systemic regulation of APX, DHAR, and GR after wounding solely the two lowest leaves. Columns represent means (±SD, $n = 5$). Asterisks indicate significant differences from control (C) samples (Student's t-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$).

ROS in the EFP

Down-regulation of the antioxidant system often correlates with ROS accumulation and subsequent oxidative modifications of proteins and lipids. However, using four different assays based on Amplex Red, H_2DCF (Harrach et al., 2008), Xylenol Orange/sorbitol (Gay and Gebicki, 2000) or dimethylaminobenzoic acid (DMAB) in conjunction with methylbenzothiazoline hydrazone (MBTH) (Okuda et al., 1991; Veljovic-Jovanovic et al., 2002) we did not detect ROS in the EFP after leaf squeezing. There was no general rise in protein oxidation while carbonylation of PP2 was even transiently decreased at 6–24 h after wounding (Gaupels et al., 2012). Moreover, formation of malondialdehyde as an indicator for lipid oxidation and oxidative stress did not show strong alterations (**Supplemental Figure 1**). From these results it can be concluded that ROS do not accumulate to detectable levels in the EFP of stressed pumpkin. Most likely, ROS are efficiently scavenged by ascorbate and glutathione, which decline after wounding but are still present at high concentrations. In the next chapters we will also present evidence suggesting that superoxide reacts with NO resulting in peroxynitrite formation and protein nitration.

Collectively, the presented data argue for wound-induced systemic redox signaling in the EFP. Future, work will reveal more details on the fate and functions of ROS during stress responses of the EFP.

NO-mediated Protein S-nitrosylation is Decreased during the SWR

ROS, NO, and its oxo-derivatives cooperate in stress-induced redox signaling (Groß et al., 2013). Dependent on the redox status NO derivatives exhibit very different chemical properties. NO reacts with superoxide at high rate constant in the course of radical-radical scavenging resulting in the formation of $ONOO^-$, which can oxidize and nitrate proteins (Pryor et al., 2006; Gaupels et al., 2011). Tyrosine (Tyr) and tryptophane residues are particularly amenable to nitration through attachment of a NO_2 group. By contrast, NO itself and the derivative N_2O_3 rather

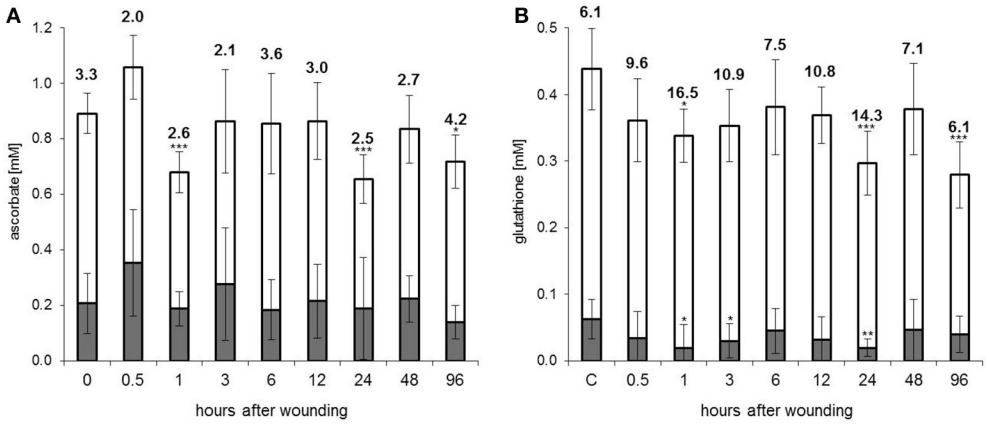

FIGURE 2 | Antioxidant levels in the EFP are decreased after leaf damage. (A) Ascorbate and **(B)** glutathione concentrations in phloem latex. White sections of the columns represent the reduced fractions of ascorbate (AsA) and glutathione (GSH). Oxidized ascorbate (DHA) and glutathione (GSSG) are shown in gray. Numbers are the AsA/DHA and GSH/GSSG ratios. Columns represent means (±SD, $n = 7$–9). Asterisks indicate significant differences from control (C) samples (Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$).

modify peptides by cysteine (Cys) S-nitrosylation (NO group; Astier et al., 2012; Yu et al., 2014).

The tripeptide glutathione has a Cys which is very sensitive to oxidation and is responsible for disulfide bond formation between two GSH molecules resulting in GSSG formation upon oxidation. This Cys is efficiently S-nitrosylated and S-nitrosoglutathione (GSNO) is a well-known reservoir of NO in plants and animals (Yu et al., 2014). NO homeostasis is under control of the enzyme GSNO reductase (GSNOR). In EFP samples S-nitrosothiol (SNO) levels were significantly down-regulated already at 30 min reaching about 50% of control levels at 6–48 h after leaf wounding (**Figure 3A**). The employed NO analyzer (NOA) cannot discriminate between protein- and low-molecular weight SNOs including GSNO. However, given the rather high GSH concentrations it can be assumed that GSNO accounts for a large fraction of total SNOs in the EFP.

For investigating protein S-nitrosylation we used the biotin switch method, which is based on replacing the SNO group by biotin. With anti-biotin antibodies it was then possible to detect candidate S-nitrosylated phloem proteins. In accordance with the NOA results, the biotin switch approach revealed a decline in S-nitrosylation of phloem proteins after wounding. Seven bands of approx. 16, 18, 29, 37, 50, 75, and 80 kD were visible in EFP samples from untreated pumpkin plants but gradually weakened at 3 and 24 h after leaf injury (**Figure 3B**). The 16- and 18 kD bands represent 16 kD Phloem Protein 1 (PP16-1) and Cyclophilin 18 (CYP18). Both proteins are wound-inducible in the EFP (Gaupels et al., 2012). The candidate S-nitrosylated ∼50 kD protein is most likely an isoform of the Phloem Protein 2 (PP2) dimer. The dimer is stable in the non-reducing buffers used for the biotin switch technique, whereas mainly monomers of the PP2 isoforms are visible after reducing SDS-PAGE (Read and Northcote, 1983; Walz et al., 2004). A more detailed time course experiment revealed that S-nitrosylation was unchanged or even slightly increased at 0.5 and 1 h but clearly diminished between 6 and 48 h after leaf wounding (**Figure 3C**). Thus, at the later time points protein S-nitrosylation decreased with similar kinetics like the total SNO levels.

The specificity of the approach was validated by treating phloem samples with 1 mM GSH or 0.5 and 1 mM GSNO. GSH is a scavenger whereas GSNO is a donor of S-nitrosylation. Accordingly, basal S-nitrosylation in the EFP samples was strongly enhanced by GSNO but reduced by GSH pre-treatment (**Figure 3D**). The shift in band pattern upon GSH treatment probably reflects S-glutathionylation of PP16-1, CYP18, and PP2. Only very weak background signals were visible in the non-biotinylated control sample. It is noteworthy that the same set of proteins was S-nitrosylated *in vivo* as well as *in vitro* after GSNO treatment implying that these EFP proteins are major targets of NO binding.

Isolation of biotinylated proteins by streptavidin-coated magnetic beads facilitated the identification of candidate S-nitrosylated phloem proteins by mass spectrometry (MS) (**Figure 3E**, **Supplemental Table 1**). Also in this experiment S-nitrosylation decreased in EFP exudates from wounded pumpkin plants and in GSH-treated samples but increased after addition of GSNO. We used 8 mM GSNO in order to maximize protein yields before further analysis by MS. Except for PP16-2 all 9 identified proteins had at least one Cys residue as a prerequisite for S-nitrosylation. PP16-2 shares 91% sequence homology with PP16-1 but lacks the two Cyss near the N-terminus. Accordingly, PP16-2 was never detected in anti-biotin western blots (WBs). For the streptavidine-based pulldown of SNO-proteins we used high-salt but otherwise non-denaturating conditions. Therefore, it is plausible that PP16-2 was co-isolated with a biotinylated protein—most likely PP16-1. Alternatively, non-biotinylated PP16-2 efficiently binds to streptavidine in an unknown fashion. PP16 proteins are phloem-mobile RNA carriers (Xoconostle-Cázares et al., 1999). Therefore, S-nitrosylation of PP16-1 could affect RNA signaling in the phloem.

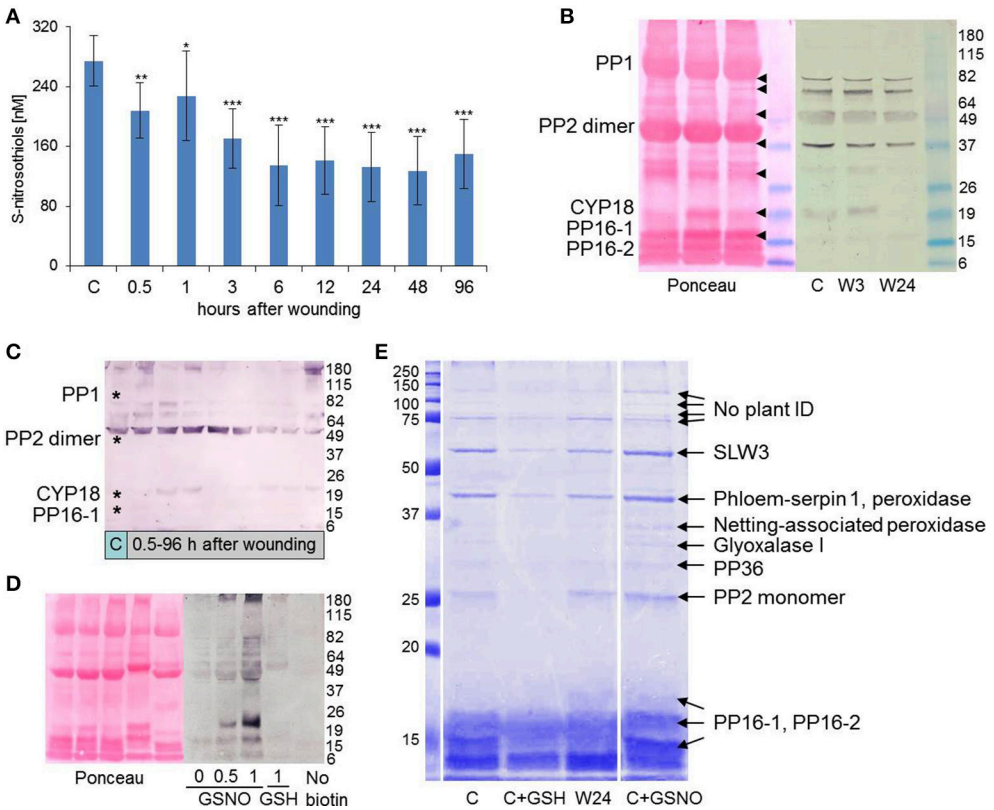

FIGURE 3 | Cysteine S-nitrosylation is reduced in the EFP after leaf wounding. (A) Total S-nitrosothiols were determined using a Sievers Nitric Oxide Analyzer. Columns represent means (±SD, $n = 7$–11). Asterisks indicate significant differences from control (C) samples (Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$). **(B–D)** Anti-biotin western blot (WB) analyses for detection of S-nitrosylated EFP proteins after biotinylation of S-nitrosylated proteins by the biotin switch assay. Successful protein transfer was confirmed by Ponceau Red staining of the nitrocellulose membrane. Molecular weight (kD) of protein standards are indicated on the right. **(B)** Comparison of protein S-nitrosylation between phloem sap from untreated control pumpkin plants (C) and wounded plants (W3, W24, 3, and 24 h after wounding). Major phloem proteins are labeled (cf. Walz et al., 2004). Note that the non-reducing buffers used for the WB facilitated the formation of PP2 dimers (cf. Read and Northcote, 1983). Arrow heads indicate positions of WB signals on the Ponceau Red stained membrane. **(C)** Time course of protein S-nitrosylation after wounding. Asterisks mark the positions of major phloem proteins. **(D)** Effect of 0.5 and 1 mM GSNO and 1 mM GSH on S-nitrosylation. One non-biotinylated control is shown. **(E)** Isolation, separation and identification of S-nitrosylated EFP proteins. Proteins identified in cut bands are indicated. Phloem sap from control plants (C) was treated or not with 8 mM GSH or 8 mM GSNO for maximal protein yield. Note that S-nitrosylation is decreased in phloem sap from a wounded plant (W24). Protein standards are labeled on the left (kD).

Both in the anti-biotin WB as well as in the streptavidine-pulldown a 26.5 kD rather than the major 24.5 kD isoform of PP2 was primarily modified by NO. Although, PP2 isoforms were described earlier (Read and Northcote, 1983; Walz et al., 2004) it is still not clear if they differ in sequence or in post-translational modifications. PP2 has 6 Cys and our data imply that at least one Cys is more amenable to S-nitrosylation in the 26.5 kD isoform compared to the 24.5 kD isoform. Reported functions of PP2 are related to the polygalacturon-binding lectin domain, RNA transport and redox-controlled formation of phloem filaments together with PP1 (Read and Northcote, 1983; Dinant et al., 2003). If these functions are modulated by S-nitrosylation remains to be investigated in a future study. Peroxidases are further redox sensitive proteins in the EFP. Both type III phloem peroxidases identified by the biotin switch technique contain several Cys residues within or near the catalytic center, which could play a role in regulation of enzyme activity by redox modifications including S-nitrosylation. To date, nothing is known about a possible NO-related regulation of the EFP proteins glyoxalase I, SLW3 (β-glucosidase), serpin-1 (proteinase inhibitor), and PP36 (cytochrome b5 reductase).

CYP18 was not identified in the streptavidin-pulldown probably because it is strongly expressed only at 0.5–6 h after wounding. No clear 18 kD band was visible at the 24 h time point and only a single peptide of CYP18 was found by MS (**Figure 3E**, **Supplemental Table 1**). In contrast, S-nitrosylated CYP18 was reproducibly detected by anti-biotin-WB at early time points after wounding and after GSNO treatment (**Figures 3B–D**). CYP18 has conserved Cys residues at sequence position 40 and 168. Disulfide bond formation between these Cys in the citrus (*Citrus sinensis*) cyclophilin CsCYP disrupted peptidyl-prolyl *cis-trans* isomerase activity and binding to the specific interaction partners thioredoxin and RNA polymerase II, thereby modulating gene transcription (Campos

et al., 2013). S-nitrosylation of plant cyclophilins has been reported for CYP20-3 of Arabidopsis (Lindermayr et al., 2005) further supporting the assumption that S-nitrosylation of Cys-40 and -168 could impact on CYP18 functions during the SWR.

Mechanical injury triggered a rise in total SNOs in leaves of pea (*Pisum sativum*) and hypocotyls of sunflower (*Helianthus annuus*; Corpas et al., 2008; Chaki et al., 2011). GSNO levels were concomitantly increased in vascular tissues as visualized by immuno-localization with anti-GSNO antibodies. Contrary to these findings high basal SNO levels and protein S-nitrosylation were systemically decreased in the EFP after leaf wounding. High SNO levels could be explained by the hypoxic conditions prevailing in the phloem, which is deeply embedded in other tissues (van Dongen et al., 2003). Oxygen deficiency is a well-known stimulus for NO production (Perazzolli et al., 2004). The damage-induced reduction in SNO abundance could be caused either by down-regulated production or increased degradation of NO/SNO within the EFP. Preliminary analyses of rape seed (*Brassica napus*) phloem exudates confirmed the wound-induced decrease in protein S-nitrosylation suggesting that this stress response is not specific for the pumpkin EFP.

Protein Tyrosine Nitration is Enhanced during the SWR

According to a current model Tyr nitration is a highly efficient radical-radical reaction between NO_2 and ROS-generated protein Tyr radicals (Pryor et al., 2006). Thus, nitroTyr formation is indicative for the simultaneous presence of NO and ROS in stressed tissues. Protein Tyr nitration can be immuno-detected by anti-nitroTyr antibodies. However, nitration is a rare event and was only occasionally detected in EFP exudates by WB analysis after SDS-PAGE. Therefore, we analyzed cumulative nitration of phloem proteins after dot blot onto a nitrocellulose membrane (**Figure 4A**). Nitration of EFP proteins gradually increased peaking at 48 h after leaf wounding. In some experiments nitrated proteins accumulated already at the 0.5 and 1 h time points. Pre-incubation of the WB membrane with the de-nitrating compound dithionite before immuno-detection by an anti-nitroTyr-antibody resulted in almost completely abolished WB signals although phloem proteins were still present on the membrane as confirmed by Ponceau Red staining. This demonstrates that the employed monoclonal antibody was specific for tyrosine nitration. Using the same approach we observed nitration of EFP proteins after watering of plants with H_2O_2 (Gaupels et al., 2008) as well as after UV-B-, ozone-, and salt stress (unpublished results). Therefore, it can be speculated that $ONOO^-/NO_2$-mediated nitration is involved in the general stress response of the EFP.

For enrichment, isolation and subsequent identification of nitrated phloem proteins we employed an anti-nitroTyr antibody coupled to agarose beads. This way, we identified the candidate nitrated phloem proteins PP16-1/-2, CYP18 (detected in the same cut band) PP2, Phloem Calmodulin-like-domain Protein Kinase 1 (PCPK1), and PP1 (**Figure 4B**, **Supplemental Table 2**). Tyr nitration of the latter three proteins was enhanced at 48 h after leaf damage whereas the band intensity for PP16-1/-2 and CYP18 was not strongly altered. CDPK1 is a particularly interesting protein because it has been shown that various kinases can be regulated by Tyr nitration. For instance, human Protein Kinase G-1α was inhibited by nitration of Tyr247 whereas the activity of Protein Kinase Bα was enhanced after nitration of Tyr350 (Rafikov et al., 2013; Aggarwal et al., 2014). Tyr nitration was observed in various plants exposed to biotic as well as abiotic stresses including mechanical injury (Corpas et al., 2008; Chaki et al., 2011). However, physiological effects of protein nitration in wounded plants were not yet investigated.

Leaf Wounding Triggers cGMP Signaling in the EFP

In response to stress cGMP is produced by soluble or membrane/receptor guanylate cyclases (GCs) (Gaupels et al., 2011). Two independent methods were employed for

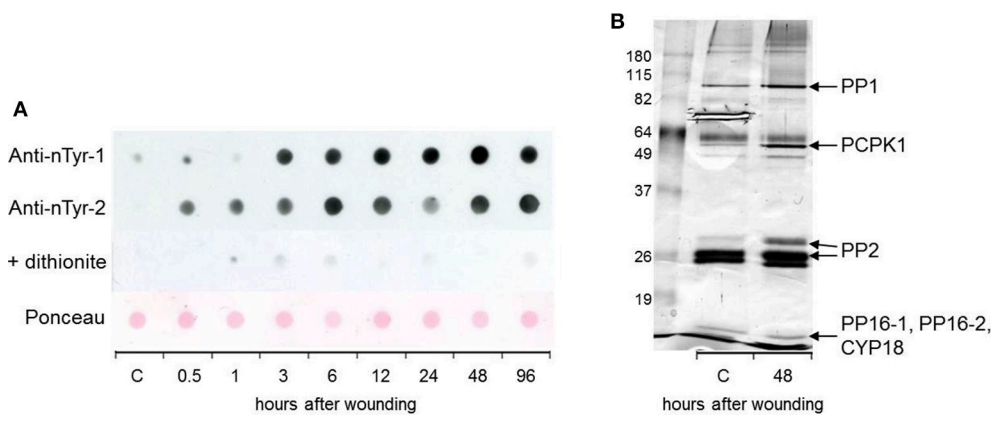

FIGURE 4 | Tyrosine nitration of EFP proteins is enhanced in response to leaf damage. (A) Nitration was detected by anti-nitrotyrosine antibodies after dot-blot transfer of total EFP proteins to a nitrocellulose membrane. Two representative experiments are shown. A duplicate membrane of experiment 2 was incubated in 100 mM dithionite for de-nitration. Ponceau Red staining of the same membrane confirmed that phloem proteins were still present after dithionite treatment. **(B)** Immuno-precipitation and identification of tyrosine-nitrated EFP proteins. Proteins were silver-stained. C, control.

determination of cGMP levels in EFP exudates—a commercial enzyme linked immunosorbent assay (ELISA) kit based on specific antibodies against cGMP and a high performance liquid chromatography (HPLC)-MS/MS system optimized for measurements of cyclic nucleotides (Bähre and Kaever, 2014). ELISA is very sensitive and allowed for the detection of 1.8 nM basal cGMP levels, which increased to 2.7 nM at 3 h and 3.8 nM at 24 h after wounding (**Figure 5A**). The induction of cGMP signaling was confirmed by HPLC-MS/MS. cGMP control samples were below the detection limit of this technique but reached 1.5 nM at the 24 h time point (**Figure 5B**). Hence, both measurements showed that cGMP was present in the EFP at low nanomolar concentrations and cGMP synthesis was systemically up-regulated after leaf damage.

Injured leaf tissues undergo programmed cell death for protecting the plant from microbial ingress. Experiments with pelargonium and sweet potato (*Ipomoea batatas*) revealed a dual role of NO in onset and confinement of wound-induced cell death. Suicide is initiated by combined enzymatic ROS production and NO-mediated inhibition of antioxidant enzymes (Arasimowicz et al., 2009). However, tissues near the damaged leaf area were protected from cell death by enhanced expression of APX and superoxide dismutases, which was dependent on NO and down-stream signaling by cGMP as uncovered by use of GC inhibitors (Lin et al., 2011). Independent of NO cGMP induces expression of the microRNA miR828 after leaf wounding (Lin et al., 2012). cGMP production by soluble GCs in animal cells is activated by binding of NO to the protein haem domain through metal nitrosylation. In plants, the Arabidopsis NO-inducible GC 1 (NOGC1) is involved in regulating the stomatal aperture. The available literature shows that cGMP is not always down- but can also be up-stream of NO and ROS in plant stress responses (Gaupels et al., 2011). For instance, the oxidative burst and subsequent induction of genes coding for antioxidant enzymes was suppressed by an inhibitor of cGMP synthesis in cadmium-stressed pea plants (Romero-Puertas et al., 2007). The exact mode of interaction of cGMP with NO and redox signals in the EFP will be deciphered in a future study.

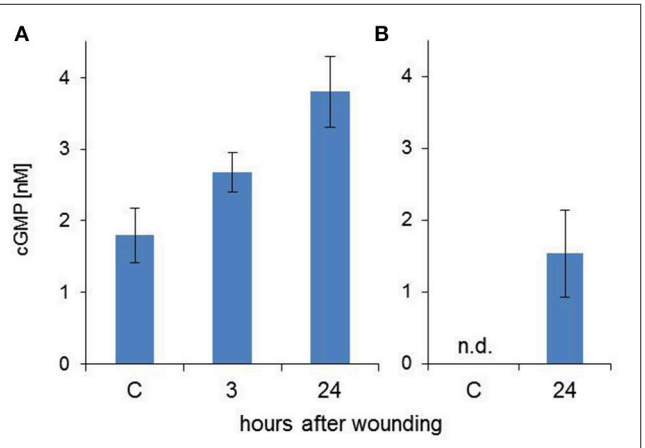

FIGURE 5 | Wounding induces cGMP signaling in the EFP. cGMP concentrations were measured using a commercial ELISA kit **(A)** or by a HPLC-MS/MS system **(B)**. Columns represent means (±SD, $n = 7$ for **A**, $n = 3$ for **B**).

CONCLUDING REMARKS

Leaf damage triggered a systemic wound response in the pumpkin EFP. Within 30–60 min the core antioxidant system of the EFP was rapidly down-regulated. We hypothesize that this would likely promote the accumulation of ROS such as O_2^- and H_2O_2 (**Figure 6**). NO-dependent protein modifications appeared to be altered with somewhat slower kinetics. The total SNO content and protein S-nitrosylation decreased whereas protein Tyr nitration increased after leaf injury. This finding cannot be explained by a general regulation of NO production or degradation. More likely, severe stress induced a shift in the composition of reactive nitrogen species away from NO to its nitrating oxo-derivatives $ONOO^-$ and NO_2. In such a scenario leaf wounding causes a drop in the EFP's

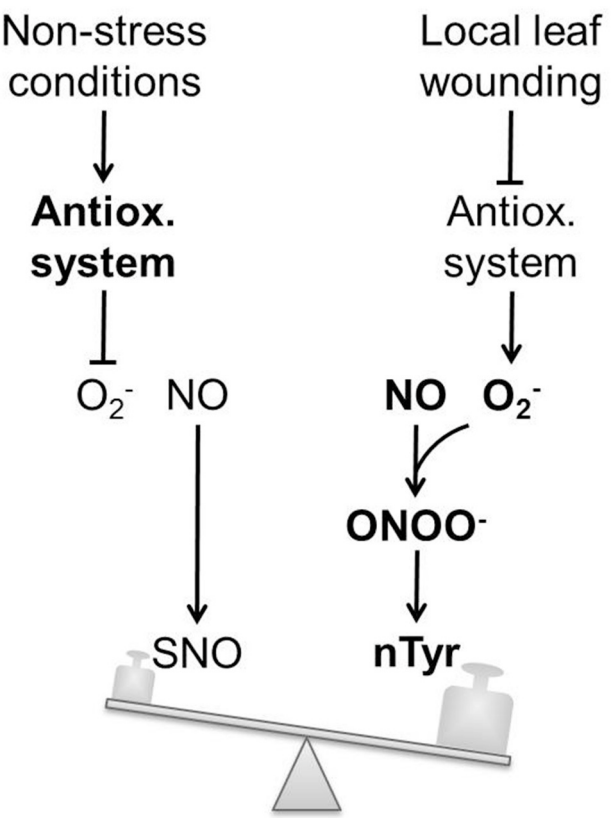

FIGURE 6 | Balance model of wound-induced NO- and redox-signaling in the EFP. Under non-stress conditions low levels of O_2- levels are maintained by the antioxidant system. Basal NO production in the EFP causes protein S-nitrosylation. Local leaf wounding triggers a systemic inhibition of the antioxidant system. Subsequent simultaneous accumulation of O_2^- and NO facilitates $ONOO^-$ formation and tyrosine nitration. Hence tyrosine nitration is an indirect evidence for both O_2^- as well as NO synthesis within the pumpkin EFP. O_2^- has a very high affinity for NO. Therefore, tyrosine nitration is favored over S-nitrosylation after wound-induced O_2^- accumulation. SNO, S-nitrosothiols; nTyr, nitrotyrosine.

antioxidant capacity and in subsequent accumulation of ROS. Particularly, O_2^- would efficiently scavenge NO to give $ONOO^-$. This reaction has a higher rate constant than the binding of NO to GSH or protein thiols. Therefore, nitration would be favored over S-nitrosylation under oxidative stress conditions (**Figure 6**). Increased protein nitration is also an indirect evidence for the simultaneous accumulation of ROS (facilitated by the inhibition of the antioxidant system) and NO. Another outcome of our work is that PP16-1, CYP18, and the 26.5 kD isoform of PP2 are redox-sensors in the EFP, which can be carbonylated, S- glutathionylated, S-nitrosylated, or tyrosine nitrated. Hence, dependent on the levels and composition of redox signals in the EFP proteins are modified by different mechanisms with specific implications for protein conformation and function. Future, work will focus on deciphering the interactions between the antioxidant system, NO and cGMP as well as the effect of redox modifications on phloem proteins.

AUTHOR CONTRIBUTIONS

FG, AF, MZ, and FC analyzed the antioxidant system. FG, AB, JK, and HS identified NO-modified proteins. VK measured cGMP. FG, KK, and JD planned the experiments. FG wrote 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.2016.00154

Supplemental Figure 1 | Lipid oxidation as an indicator for oxidative stress does not increase after leaf wounding. Lipid oxidation in EFP exudates was determined by the TBARS assay. Columns represent means (±SD, $n = 9$).

Supplemental Table 1 | Identification of candidate S-nitrosylated proteins in EFP latex.

Supplemental Table 2 | Identification of candidate tyrosine nitrated proteins in EFP latex.

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ABA Suppresses *Botrytis cinerea* Elicited NO Production in Tomato to Influence H_2O_2 Generation and Increase Host Susceptibility

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Abscisic acid (ABA) production has emerged a susceptibility factor in plant-pathogen interactions. This work examined the interaction of ABA with nitric oxide (NO) in tomato following challenge with the ABA-synthesizing pathogen, *Botrytis cinerea.* Trace gas detection using a quantum cascade laser detected NO production within minutes of challenge with *B. cinerea* whilst photoacoustic laser detection detected ethylene production – an established mediator of defense against this pathogen – occurring after 6 h. Application of the NO generation inhibitor *N*-Nitro-L-arginine methyl ester (L-NAME) suppressed both NO and ethylene production and resistance against *B. cinerea*. The tomato mutant *sitiens* fails to accumulate ABA, shows increased resistance to *B. cinerea* and we noted exhibited elevated NO and ethylene production. Exogenous application of L-NAME or ABA reduced NO production in *sitiens* and reduced resistance to *B. cinerea.* Increased resistance to *B. cinerea* in *sitiens* have previously been linked to increased reactive oxygen species (ROS) generation but this was reduced in both L-NAME and ABA-treated *sitiens.* Taken together, our data suggests that ABA can decreases resistance to *B. cinerea* via reduction of NO production which also suppresses both ROS and ethylene production.

Keywords: nitric oxide, reactive oxygen species, ethylene, ABA, *sitiens*, *Botrytis cinerea*

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INTRODUCTION

The outcome of pathogen interactions with plants is governed by multiple events (reviewed by Jones and Dangl, 2006). Initial contact involves host detection of pathogen-associated molecular patterns [PAMPs, microbial-associated molecular patterns; also referred to as (MAMPs)] to initiate PAMPs-triggered immunity (PTI). Pathogens have evolved a range of effectors which can suppress PTI, some of which may be recognized by the host–classically by Resistance (*R*) genes – to initiate effector-triggered immunity (ETI) which can lead to the form of programmed cell death known as the hypersensitive response (HR; Mur et al., 2008). Superimposed on these interactions are the various pathogen-derived toxins, enzymes, and host modifying proteins that can drive symptom development. Based on disease symptoms it is possible to crudely classify pathogens into biotrophs (parasitizing living host tissue) or necrotrophs (predating death host tissue) or those exhibiting

combinations of both lifestyles – hemibiotrophs (Schulze-Lefert and Panstruga, 2003; Oliver and Ipcho, 2004; Oliver and Solomon, 2010).

Pathogen-associated molecular patterns-triggered immunity and ETI-linked defense gene expression is co-ordinated by a large number of hormones (Robert-Seilaniantz et al., 2007). Traditionally, salicylic acid (SA) mediated defenses have been linked to defense against biotrophs and jasmonates (JA)/ethylene (Et) against necrotrophs (Oliver and Solomon, 2010) although this represents a considerable over-simplification (Robert-Seilaniantz et al., 2011). Nitric oxide (NO) should also be considered as a major defense initiator (Mur et al., 2013). Thus, NO has been shown to be rapidly generated in plants following challenge with biotrophic and necrotrophic pathogens (Delledonne et al., 1998; Foissner et al., 2000; Mur et al., 2005, 2012; Prats et al., 2005; Besson-Bard et al., 2008). Despite many mechanisms of NO generation being proposed, it appears that under aerobic conditions NO is mostly produced by cytosolic nitrate reductase (NR) acting on NO_2^- as a nitrite reductase (Gupta et al., 2011). NO production has been linked to PTI, the HR and the formation of defense associated cell-wall appositions (Zeidler et al., 2004; Prats et al., 2005; Mur et al., 2008). Further, the importance of NO to plant defense has been shown through the use of chemical inhibitors, transgenic lines, or differential N fertilizer regimes to modify NO production and impact on the degree of resistance (Delledonne et al., 1998; Boccara et al., 2005; Mur et al., 2012; Gupta et al., 2013). The mechanisms through which NO impacts on defense signaling cascades have also been extensively examined, with reversible *S*-nitrosylation of proteins and irreversible tyrosine nitration of key components emerging as important regulatory events (Cecconi et al., 2009; Leitner et al., 2009). Notable examples of the role of *S*-nitrosylation in defense are the modification of the vital SA signaling component NON-EXPRESSOR OF PATHOGENESIS-RELATED PROTEIN1 (NPR1; Tada et al., 2008) and the reactive oxygen species (ROS) generating complex NADPH oxidase, AtRBOHD (Yun et al., 2011). Further, there is clear evidence that NO, ROS, and SA pathways work together to establish a systemic acquired resistance (SAR) that can be exhibited by the whole plants following the localized initiation of resistance (Wang et al., 2014). Such examples demonstrate the close interactive role of NO with other signaling pathways which also include JA and Et (Mur et al., 2013).

Abscisic acid (ABA) is classically associated with plant responses to abiotic stress particularly drought (Shinozaki and Yamaguchi Shinozaki, 1996) but is now well recognized to also play roles in plant responses to pathogens (Cao et al., 2011). However, rather than aiding resistance, ABA, appears to be mostly contributing to susceptibility. Thus, exogenous application of ABA increased the susceptibility of *Arabidopsis* to *Pseudomonas syringae* pv. *tomato* (Fan et al., 2009) or of rice to the rice blast pathogen *Magnaporthe grisea*; in this latter case even with avirulent strains (Jiang et al., 2010). Correspondingly, *Arabidopsis* ABA biosynthetic mutants exhibit enhanced resistance to *P. syringae* (de Torres-Zabala et al., 2007; Goritschnig et al., 2008). Considering mechanisms through which ABA antagonisms act, one mechanism appear to perturbation of SA signaling. Some key studies have shown that ABA suppresses expression of the SA biosynthetic gene *ISOCHORISMATE SYNTHASE1* (*ICS1*) in *Arabidopsis* (Yasuda et al., 2008) and (e.g.) *NPR1* in rice (Jiang et al., 2010). Other workers have shown a reciprocal antagonism with the increased disease resistance in the *Arabidopsis* mutant *cpr22* at least partially linked to SA-mediated perturbation of ABA signaling (Moeder et al., 2010; Mosher et al., 2010). However, ABA impacts are not only associated with SA-mediated defenses – which are mostly deployed against biotrophs. ABA effects can also be seen in initiating ROS generation with latter contributing to ABA signaling (Pei et al., 2000). Similarly, ABA increase NO generation which can increase the activity of antioxidant enzymes (Zhou et al., 2005). Such observations suggest a subtle interaction between ABA, ROS, and NO.

Abscisic acid treatment could also compromise the resistance of *Arabidopsis* to the necrotroph *B. cinerea* (AbuQamar et al., 2006). Much work on the interaction of ABA and *Botrytis* has focused on the tomato mutant *sitiens* (Asselbergh et al., 2007). *sitiens* is deficient in ABA production through a mutation in the biosynthetic ABA-aldehyde oxidase (Harrison et al., 2011). *sitiens* exhibits considerably increased resistance to *B. cinerea* and this has been variously linked to increased SA effects (Audenaert et al., 2002) and/or epidermal hydrogen peroxide generation with cell wall modifications (Asselbergh et al., 2007). A similar link between ABA and suppression of ROS during attack by *B. cinerea* has been made in *Arabidopsis* (L'Haridon et al., 2011). Given such observations it is unsurprising that *B. cinerea* strains can encode genes for ABA biosynthesis (Gong et al., 2014).

In this current study, we examine the interaction between ABA and NO generation in tomato. Through the use of pharmacological inhibitors of NO generation we demonstrate the importance of NO to tomato defense against *B. cinerea.* Exploiting online, *in planta* measurements, we show a greatly elevated NO generation in the ABA deficient mutant *sitiens*. Suppression of NO production in *sitiens*, with NO generation inhibitors or the exogenous application of ABA, also compromised the increased resistance in this mutant to *B. cinerea*. Staining ROS production using 3,3′-diaminobenzidine (DAB) suggested that the levels were influenced by the rates of NO generation. We propose that ABA-influenced antagonism of NO generation could be an important mechanism through which pathogens can suppress defenses to establish compatible interactions with their host.

MATERIAL AND METHODS

Tomato Plant Growth Conditions

Tomato genotypes, cv. Ailsa Craig and *sitiens* were obtained from the Tomato Genomics Resource Centre (TGRC) at UC, Davis USA[1]. Tomato plants were cultivated in Levington Universal compost in 10 cm diameter plastic pots. Plants were maintained in Conviron (Controlled Environments Ltd, UK) growth rooms at 24°C with a light intensity of 110 μmol/m/s and a 12 h

[1]http://tgrc.ucdavis.edu

photoperiod for 4 weeks. At 4 weeks *sitiens* plants were considerably smaller than cv. Ailsa Craig but exhibited no evidence of any other defects (e.g., wilting) that were visible to the naked eye. Plants used for NO and Et measurements were transported to Radboud University (The Netherlands) via road and car ferry by the authors. Plants were then kept at Radboud University for 2 days under identical growth conditions to those in Aberystwyth to allow plant physiology to normalize prior to gas measurements being attempted.

Botrytis cinerea Culture and Storage

Botrytis cinerea (strain IMI 169558) was maintained on potato dextrose agar (PDA) in an inverted position in a Gallenkamp illuminated cooled incubator maintained at 20°C with a 12 h photoperiod. PDA (Potato extract 4.0 g L^{-1}; Glucose 20.0 g L^{-1}; Agar 15.0 g L^{-1}) was prepared according to manufacturer's instructions.

Conidia were harvested from the plate surface by flooding the plate with a carrier media of potato dextrose broth (PDB) and dislodging the conidia with an L-shaped glass rod. The density of conidia in the suspension was determined using a Neubauer Haemocytometer and the solution was diluted accordingly to 1×10^5 conidia mL^{-1} to provide a 30 mL standardized infection solution.

Four-week old tomato plants were inoculated with *B. cinerea* in one of two methods. Where trace gas detection (NO or Et) was being measured, the conidial suspension was sprayed on to plants to run-off. In all other occasions, inoculation involved application of 10 μL drops of spore solution, pipetted directly onto the adaxial leaf surface. Inoculation was made to the first four true leaves (excluding cotyledons), with seven drops of solution being applied to three leaflets per leaf with a total of 84 drops per plant.

Chemical Treatment of Tomato Plants

In wild type cv. Ailsa Craig plants, chemicals that would alter NO production were applied through the excised petioles of branches so that the entire compound leaf was treated. Selected compound leaves represented the oldest true leaves of 4-week-old plants. Excised branches were placed in 100 mL beakers with 50 mL of either 0.1 mM sodium nitroprusside (SNP, an NO donor) or 5 mM *N*-Nitro-L-arginine methyl ester (L-NAME; an inhibitor of NO generation) or *N*-Nitro-D-arginine methyl ester (D-NAME; a biologically inactive isomer of L-NAME) for 24 h prior to infecting the leaves with *B. cinerea*. Excised branches in beakers were maintained at 24°C with a light intensity of 110 μmol/m/s and a 12 h photoperiod before and after spot-inoculation with *B. cinerea*.

Chemicals could not be applied to *sitiens* plants through cut petioles as this led to rapid tissue collapse of the leaves. Instead, chemicals were applied to intact 4-week-old *sitiens* plants as a foliar spray which included 0.2% Silwet (v/v) as wetting agent. Although this may not have allowed accurate estimations of applied dose to be determined it proved to be the only means (in our hands) to apply chemicals and maintain *sitiens* leaf viability.

Estimating Fungal Biomass by Quantitative PCR (qPCR)

DNA was extracted from eight leaf samples with single inoculations of *B. cinerea* using a DNeasy Mini Kit (Qiagen, UK) following manufacturer's instructions. DNA samples were diluted to 1 ng μL^{-1} ultrapure H_2O. Samples (25 μL) were prepared by mixing 10 μL DNA solution with 16 μL SYBR™ Green Mastermix (Applied Biosystems, UK) and primers (300 nM) for *Arabidopsis* (iASK1: CTTATCGGATTTCTCTAT GTTTGGC; iASK2: GAGCTCCTGTTTATTTAACTTGTACA TACC) to generate an 131 bp amplicon and *B. cinerea* (CG11: AGCCTTATGTCCCTTCCCTTG; CG12: GAAGAG AAATGGAAAATGGTGAG) to generate a 58 bp amplicon (Gachon and Saindrenan, 2004). PCR used a Bio-Rad ABI7300 thermocycler amplifying using the following conditions: 15 min at 95°C followed by 50 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 1 min. This was followed by a dissociation (melting curve), according to the software procedure. Serial dilutions of pure genomic DNA from each species were used to trace a calibration curve, which was used to quantify plant and fungal DNA in each sample. Results were expressed as the CG11/iASK ratio of mock to inoculated samples.

Nitric Oxide (NO) Measurements Using a Quantum Cascade Laser-Based Sensor

The configuration of the quantum cascade laser (QCL)-based sensor for NO detection is detailed elsewhere (Cristescu et al., 2008). Briefly, the QCL emitting infrared light around 1900 cm^{-1} that passes through an absorption multi-pass cell, in which the airline enters transporting the NO released by a single inoculated rosette within a glass cuvette (∼500 mL volume). The NO production is directly detected by measuring the attenuation of the laser intensity due to the NO absorption in the cell. Infected plants were placed in glass cuvette and flushed with air at a controlled continuous flow rate of 1 L h^{-1}. Multiple cuvettes could be monitored in sequence, each being measured for ∼13 min. Each experiment was repeated to give similar results and the outcomes of one representative experiment are shown.

Ethylene Measurements Using Photoacoustic Laser Spectroscopy

Ethylene production was monitored in real-time using a gas flow-through in-line system fitted with a photoacoustic laser-based ethylene detector (ETD-300, Sensor Sense BV) able to detect on-line 300 parts per trillion volume of ethylene within 5 s (Cristescu et al., 2008). Gas was regulated by an automated valve control box (VC-6, Sensor Sense B.V) and ethylene emanation from a tomato plant within a glass cuvette (254 mL volume) was alternately monitored for 15 min (5 s per acquisition point), at a controlled continuous flow rate of 1.5 L h^{-1} by flushing with air and preventing accumulation induced effects. KOH and $CaCl_2$ scrubbers were incorporated into the system to remove CO_2 and H_2O, respectively. Each experiment was repeated to give similar results and the outcomes of one representative experiment are shown.

Visualization and Quantification of H_2O_2 Generation

After sampling, the excised leaf disks were immersed in an aqueous solution of 1 mg ml^{-1} DAB (pH 3.8) and incubated at room temperature for 4 h. The leaves were removed from the DAB solution and fixed and cleared in absolute ethanol. The samples were scanned using a flat-bed scanner and the intensity of staining was quantified using Image-Quant TL, Software (GE Healthcare Life Sciences, UK).

Statistical Analyses

Data were subjected to analysis of variance using Minitab v.14 (Minitab Ltd, Coventry, UK), after which residual plots were inspected to confirm data conformed to normality. Comparisons of data points from different treatments with controls were performed using Tukey multiple pairwise comparison test. Timecourse data were compared using repeated measures anova. Differences with $P < 0.05$ were considered significant.

RESULTS

NO Production Is Tomato Is Required for Resistance to *B. cinerea*

We have demonstrated that NO contributes both to ethylene production and resistance to *B. cinerea* in *Arabidopsis* (Mur et al., 2012). To investigate the role of NO in tomato challenged with *B. cinerea*, the production of NO was measured using a QCL-based approach (**Figure 1A**). NO was rapidly produced in cv. Ailsa Craig following infection with *B. cinerea* even before it was possible – due to technical limitations – to take the first measurement at 0.5 h after inoculation. NO production was in slow decline over the subsequent 24 h period. This was specific to pathogen attack as application of PDB, used to resuspend the fungal conidia, failed to elicit increased NO production (**Figure 1A**). Ethylene production was also elicited following challenge with *B. cinerea* but maximum rates of production were achieved some 6 h after that of NO (**Figure 1B**).

To investigate the role of NO in contributing to resistance to *B. cinerea* experiments were undertaken when a NO donor (SNP) or an inhibitor of NO production (L-NAME) were applied to tomato plants. Thus, 0.1 mM SNP was applied to cut petioles of plants and after 24 h, inoculated with *B. cinerea* with lesion development assessed over a subsequent 72 h period. The developing *B. cinerea* lesions appeared to be smaller and more defined that those forming on controls at 72 h (compare **Figures 2A,B**). Conversely, if the 5 mM L-NAME – was applied to tomato leaves, *B. cinerea* lesions were highly variable in phenotype but were also larger than in control infections and occasionally coalesced to cover the entire leaf (**Figure 2C**). If 5 mM D-NAME – an inactive isomer of L-NAME – was applied to the leaves, the *B. cinerea* lesions appeared to be more similar to those forming in control cv. Ailsa Craig plants (compare **Figures 2D** with **2A**).

Measuring NO production in cv. Ailsa Craig and L-NAME-NAME fed plants suggested that the NO production was significantly ($P < 0.001$) reduced only in L-NAME-treated plants (**Figure 2E**). Correspondingly, laser photoacoustic detection indicated that maximal ethylene production was seen following 9 hpi (hour after infection) was significantly ($P < 0.001$) reduced with L-NAME treatment (**Figure 2F**). Taken together, these data suggest that NO, which may trigger ethylene production, contributes to tomato defense against *B. cinerea*.

The ABA Mutant Sitiens Displays High Level NO Production

We subsequently sought to test if NO played a role in the elevated resistance to *B. cinerea* which is exhibited by the

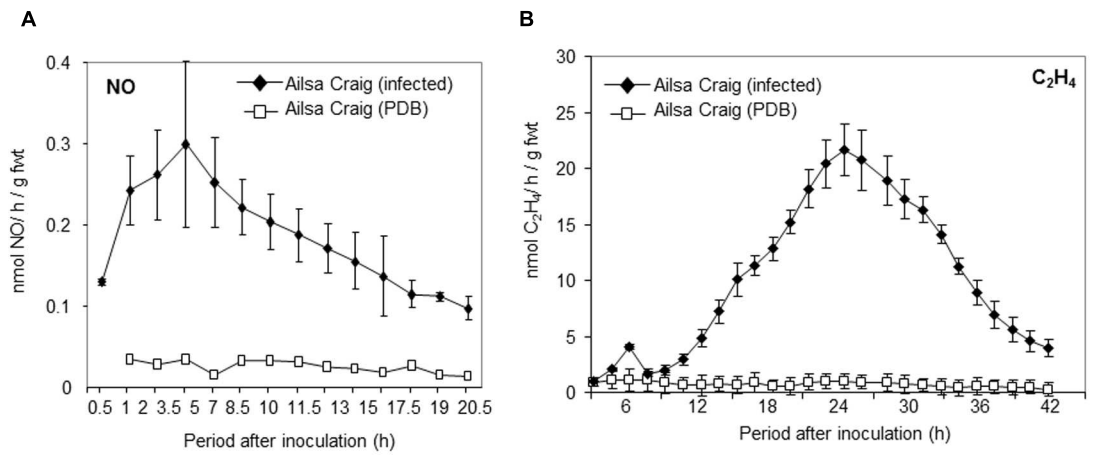

FIGURE 1 | Nitric oxide (NO) and ethylene production in tomato cultivar Ailsa Craig challenged with *Botrytis cinerea*. **(A)** NO and **(B)** ethylene were measured, following spraying whole 4 weeks old tomato Ailsa Craig plants with **(♦)** conidial suspensions of *B. cinerea* in potato dextrose broth (PDB) or **(□)** PDB alone, using, respectively, on-line quantum cascade laser (QCL) detection or photoacoustic laser detection. The presented data are the result of measurements of two *B. cinerea* challenged tomato plants and two PDB challenged tomato plants for each gas. The error bars therefore represent the range of results around a mean ($n = 2$).

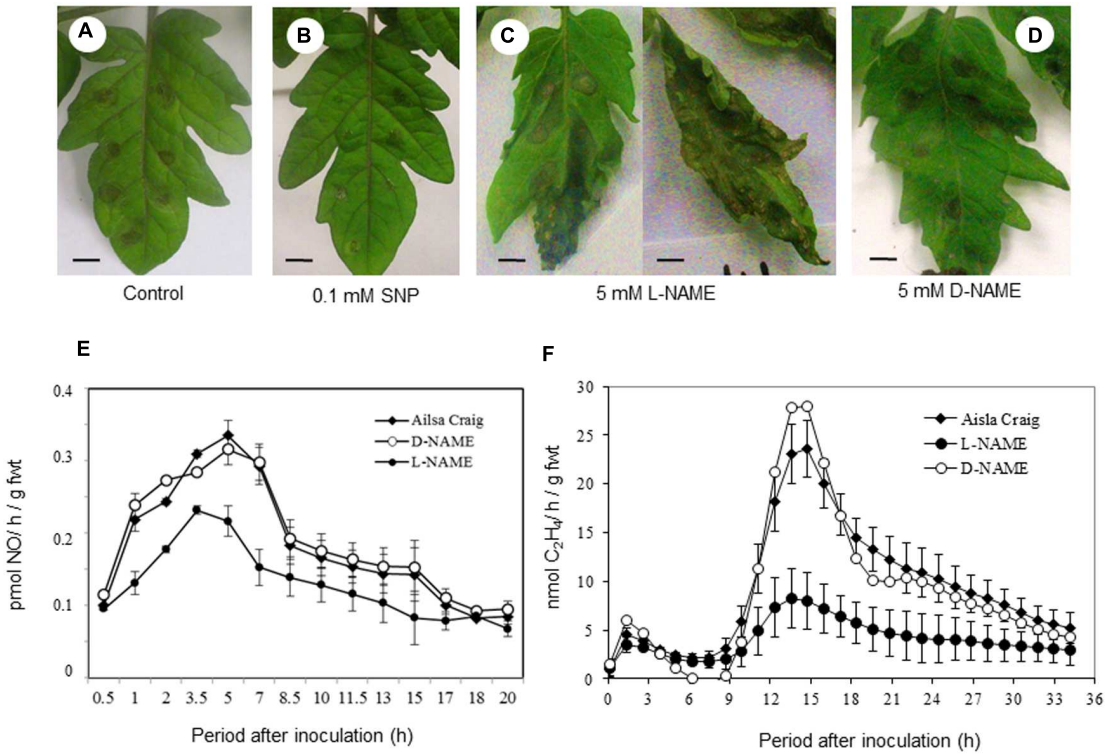

FIGURE 2 | Sodium nitroprusside (SNP) and *N*-Nitro-l-arginine methyl ester L-NAME) effects on *B. cinerea* lesion development in tomato. Excised compound leaves from 4 weeks old Ailsa Craig plants were placed in 100 mL beakers with 50 mL of either **(A)** water, **(B)** 0.1 mM SNP (an NO donor) or **(C)** 5 mM *N*-Nitro-l-arginine methyl ester (L-NAME; an inhibitor of NO generation) or **(D)** *N*-Nitro-d-arginine methyl ester (D-NAME; a biologically inactive isomer of L-NAME) for 24 h prior to infecting the leaves with drops of conidial suspensions of *B. cinerea.* Excised compound leaves were maintained at 24°C in beakers with a light intensity of 110 μmol/m/s and a 12 h photoperiod when they were spot-inoculated with *B. cinerea.* The lesions were photographed at 72 h after challenge with *B. cinerea.* Bars = 1 cm. **(E)** NO and **(F)** ethylene were measured in *B. cinerea* infected compound leaves in **(♦)** water-fed Ailsa Craig plants and plant treated with either 5 mM L-NAME **(●)** or 5 mM D-NAME **(○)**. NO and Et were measured using QCL detection or photoacoustic laser detection, respectively. The presented data are the result of measurements of two *B. cinerea* challenged compound leaves for each gas. The error bars therefore represent the range of results around a mean ($n = 2$).

ABA-biosynthetic *sitiens* tomato mutant. Measuring *B. cinerea* elicited NO in *sitiens* showed >2-fold greater rates of production compared to cv. Ailsa Craig (**Figure 3A**). The levels of NO produced in unchallenged *sitiens* plants did not significantly differ from those of cv. Ailsa Craig. *B. cinerea*-induced production of ethylene was also elevated in *sitiens* plants (**Figure 3B**).

In order to establish the importance of increased NO in *sitiens* elevated resistance, cv. Ailsa Craig and *sitiens* plants were sprayed with L-NAME. The resulting lesions appeared to be form less quickly in L-NAME sprayed plants (**Figure 4A**). Lesions forming on D-NAME treated plants did not appear different to the water treated control on either genotype (data not shown). To quantify the differences in lesion formation, *B. cinerea* fungal biomass was measured in lesions developing in cv. Ailsa Craig, *sitiens* and *sitiens* plants sprayed with either L-NAME or D-NAME at 72 hpi (**Figure 4B**). This indicated reduced fungal development in *sitiens* which was consistent with the elevated resistance seen in this line. However, fungal biomass was significantly increased following L-NAME but not D-NAME, implicating NO as a component of cv. Ailsa Craig and; crucially, *sitiens* defense against *B. cinerea.* To confirm that that L-NAME was having an effect on NO production in *sitiens*, this was assessed at 6 hpi with *B. cinerea* in a parallel experiment. This a timepoint which has previously been indicated as the displaying maximal rates of NO production (**Figures 1A** and **3A**). Spraying *sitiens* plants with L-NAME significantly ($P < 0.01$) decreased *B. cinerea* induced NO production compared to chemically untreated *sitiens* or D-NAME treated controls (**Figure 4C**). Logistical constraints prevented the effects of L/D-NAME on NO production to be assessed in cv. Ailsa Craig.

ABA Suppresses *B. cinerea* NO and ROS Production

As *sitiens* is deficient in ABA, it was hypothesized that exogenous application of ABA could reduce NO production. Thus, *sitiens* plants as well as cv. Ailsa Craig were sprayed with 100 mM ABA and then after 1 h, we challenged with *B. cinerea.* Examining the effect of ABA treatment on lesion development in *sitiens* suggested that lesion sizes at 72 hpi tended to be greater compared to untreated controls. The phenotypic effects of ABA on cv. Ailsa Craig were more difficult to assess visually (**Figure 5A**). This slight effect of ABA on *B. cinerea* infected on of cv. Ailsa Craig was also suggested from estimations of

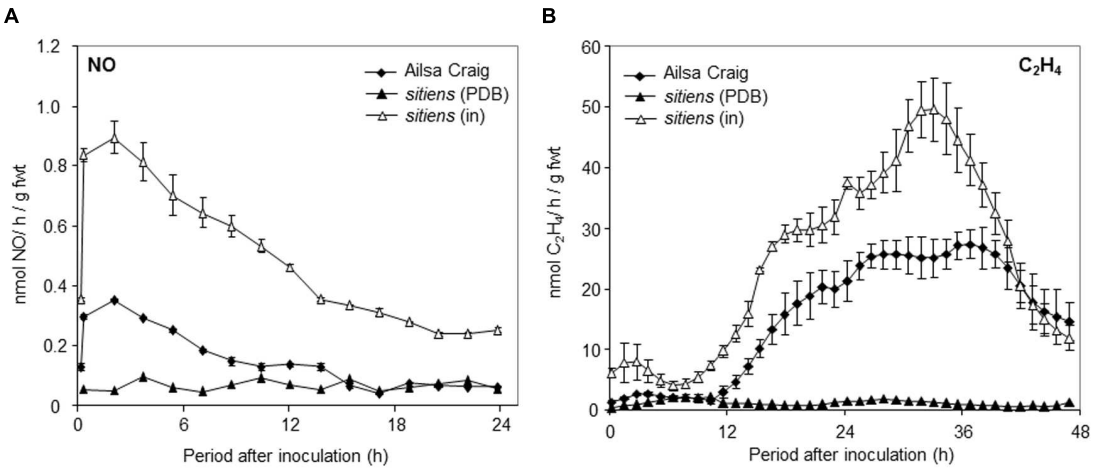

FIGURE 3 | Nitric oxide and ethylene production in *sitiens* tomato plants challenged with *B. cinerea*. **(A)** NO and **(B)** ethylene were measured, following spraying whole 4 weeks old tomato **(♦)** cv. Ailsa Craig or **(Δ)** *sitiens* plants with conidial suspensions of *B. cinerea* in PDB or **(▲)** *sitiens* plants with PDB alone using on-line QCL or photoacoustic laser for NO and ethylene detection, respectively. The presented data are the result of measurements of two *B. cinerea* challenged tomato plants and two PDB challenged tomato plants for each gas. The error bars therefore represent the range of results around a mean ($n = 2$).

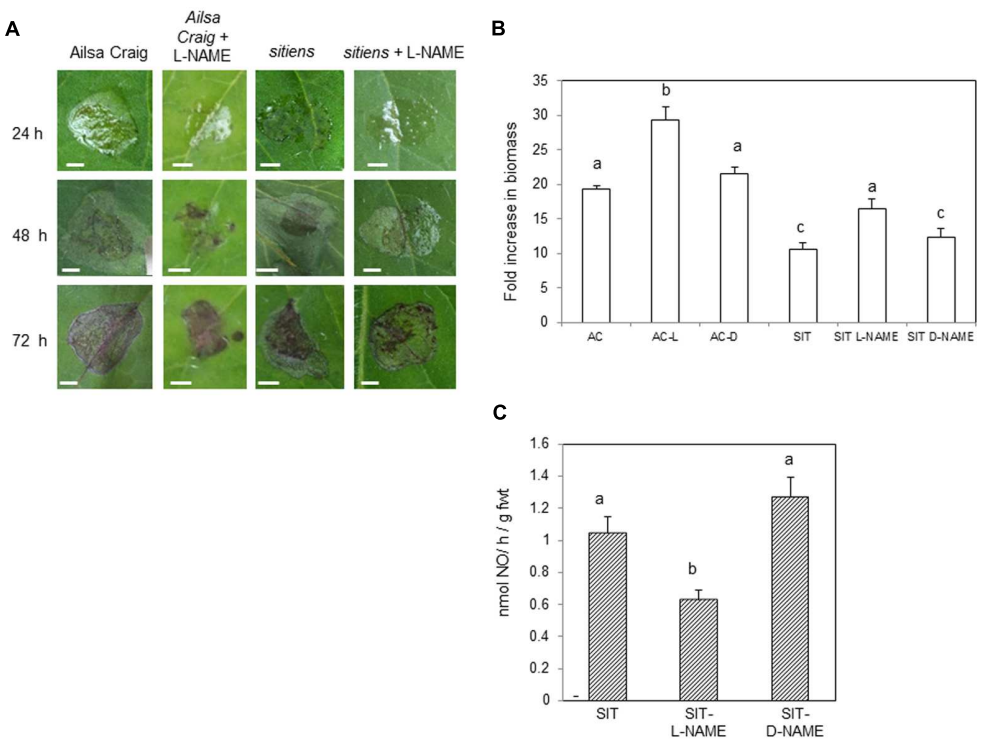

FIGURE 4 | The effect of reduced NO production on *Botrytis cinerea* infections of *sitiens*. **(A)** Representative *B. cinerea* lesion phenotypes in cv. Ailsa Craig and *sitiens* sprayed with either water or 5 mM Nitro-L-arginine methyl ester (L-NAME); an inhibitor of NO production. Note the patterns of water-soaking (tissue collapse) and necrosis (black). Bars = 0.5 cm. **(B)**. *B. cinerea* fungal DNA content in 1 cm^2 diameter cored lesions isolated from 4 weeks old control cv. Ailsa Craig (AC), *sitiens* (SIT) sprayed 1 h prior to spot-inoculation with *B. cinerea,* sprayed with either L-NAME (L) or D-NAME (D; an inactive L-NAME isomer, 5 mM Nitro-d-arginine methyl ester) sampled at 72 h post-infection. Results are expressed as fold increase in biomass compared to $t = 0$ samples. Mean measurements ($n = 5$ cores) ±SE following inoculation within *B. cinerea* are given. Significant differences are indicated with letters. **(C)** NO production measured at 6 h (peak NO production; see **Figure 1A**) following spraying conidial suspensions of *B. cinerea* of whole 4 weeks old tomato *sitiens* plants. Three treatments were assessed; untreated *sitiens* plants (SIT), plants sprayed 24 h prior to inoculation with *B. cinerea* with either L-NAME (SIT-L-NAME) or D-NAME (SIT-D-NAME). Mean NO production was measured in three plants per treatment using a QCL system. Significant differences are indicated with letters.

fungal biomass at 72 hpi (**Figure 5B**). Although there was a trend toward increased fungal development with ABA treatment differences were not significant compared to controls. This was in contrast to *sitiens* where the application of ABA significantly increased fungal biomass suggesting that resistance was being reduced.

The impact on NO on ABA in *sitiens* was measured using the QCL-based system (**Figure 5C**). Logistical constraints limited the number of samples that could be measured so only the effect of ABA on *sitiens* was determined following challenge with *B. cinerea*. NO production in the control plant was in line with that previously observed (e.g., **Figure 3A**). However, rates of NO production were reduced by ∼half in two ABA treated *sitiens* plants (**Figure 5C**).

In a seminal paper, Asselbergh et al., (2007) linked resistance in *sitiens* to elevated H_2O_2 production. We assessed if NO could also be contributing to H_2O_2 generation in cv. Ailsa Craig and *sitiens* in cores from tomato leaves at 24 h after inoculation with *B. cinerea* and stained with DAB (**Figure 6A**). Lesions with brown DAB staining were clearly observed in cv. Ailsa Craig and this appeared to be reduced by treatment with L-NAME and ABA. Greater staining was observed in *sitiens* compared to cv. Ailsa Craig and this was also reduced by L-NAME and ABA. This was further suggested when the extent of staining was measured using image analysis software (**Figure 6B**). Although a semi-quantitative measure at best, the data suggested significant reductions ($P < 0.01$) in DAB we seen in L-NAME/ABA treated cv. Ailsa Craig and *sitiens*. In the case of treatments of *sitiens* L-NAME/ABA treated plants results in patterns of DAB staining that were not significantly different from similarly treated cv Ailsa Craig samples. The data suggested that NO contributes to the elicitation of *B. cinerea* elicited H_2O_2 in wild type tomato and also to the elevated H_2O_2 observed in *sitiens*.

DISCUSSION

Nitric oxide effects on plant responses to stress have been extensive characterized using *Arabidopsis* but not exclusively so, with some important studies being undertaken in tomato, which has the virtue of being a both a model species and an important crop. Examples of studies establishing NO effects in stress tolerance in tomato include resistance to bacterial wilt (caused by *Ralstonia solanacearum*; Hong et al., 2013), root-knot nematode (*Meloidogyne incognita*; Zhou et al., 2015), Cu and Cd (Wang et al., 2015) and heat shock (Piterkova et al., 2013). Our study sought to investigate further investigate role of NO in the interaction of tomato with *B. cinerea*. Resistance to *B. cinerea* in has been shown to be influenced by Et

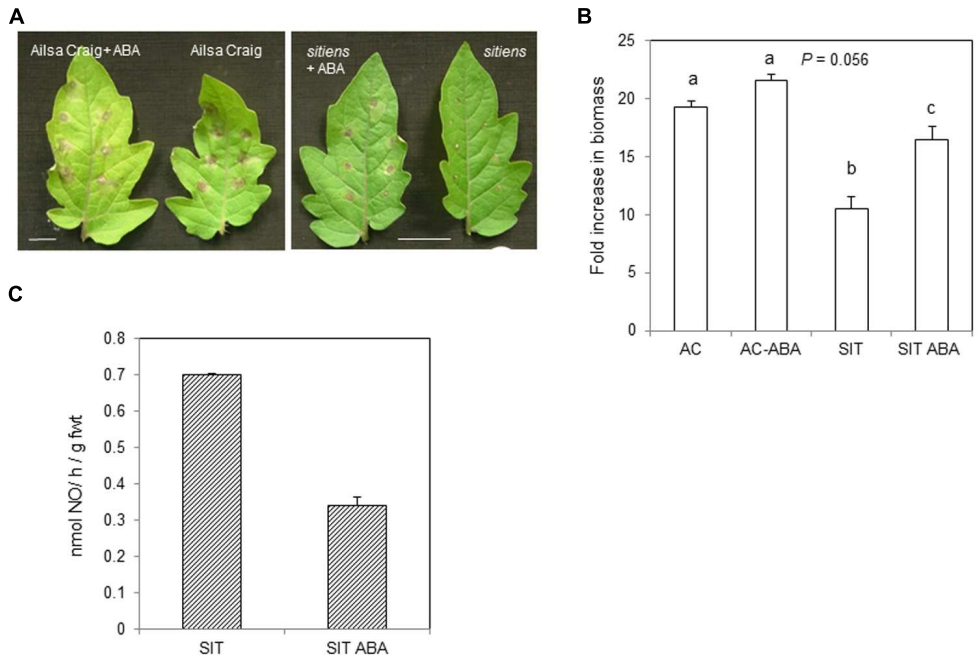

FIGURE 5 | Nitric oxide production in *B. cinerea* challenged *sitiens* treated with abscisic acid (ABA). (A) cv. Ailsa Craig or *sitiens* plants were sprayed with either 100 mL ABA in 0.2% Silwet (v/v) or with water [+0.2% Silwet (v/v)] and then multiply spot-inoculated with *B. cinerea* after 1 h. Lesion phenotypes were imaged at 72 hpi. Bars = 1 cm. **(B)** *B. cinerea* fungal DNA in cv. Ailsa Craig (AC), *sitiens* (SIT) sprayed with either 100 mL ABA in 0.2% Silwet (v/v) or with water [+0.2% Silwet (v/v)]1 h prior to spot-inoculation with *B. cinerea*. 1 cm^2 diameter cored lesions were sampled at 72 h post-infection. Results are expressed as fold increase in biomass compared to $t = 0$ samples. Mean measurements ($n = 5$ cores) ±SE following inoculation within *B. cinerea* are given. Significant differences are indicated with letters. **(C)** NO was measured, following spraying whole 4 weeks old *sitiens* tomato plants with conidial suspensions of *B. cinerea*. *sitiens* plants were either sprayed with water (SIT) or 100 mM ABA (SIT ABA, 2 repetitions) 1 h prior to challenge with *B. cinerea*. Data are presented at 6 hpi when maximal NO production has been demonstrated (see **Figures 1** and **2**). The error bar represent the range of results around a mean ($n = 2$).

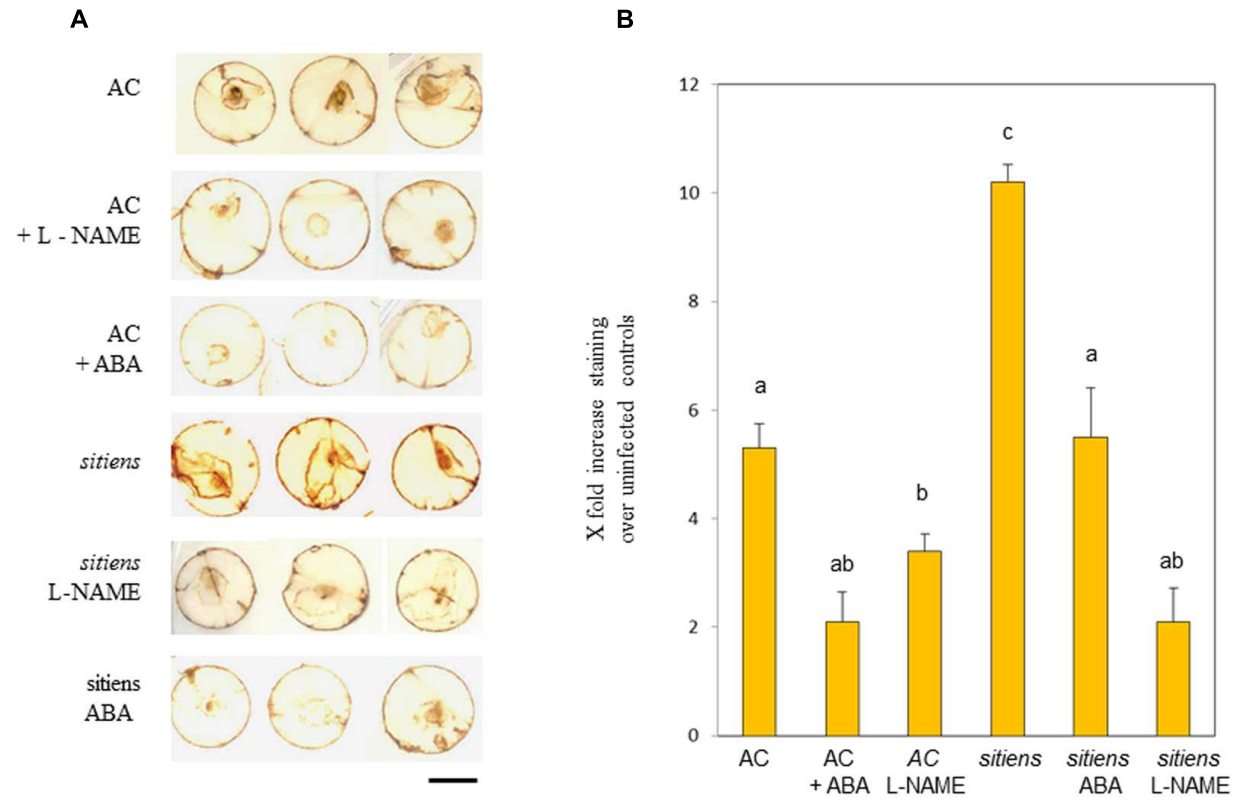

FIGURE 6 | Nitric oxide and abscisic acid (ABA) effects on *B. cinerea* elicited H_2O_2 production. (A) H_2O_2 production as detected by staining with 3,3, diaminobenzidine (DAB) in 1 cm diameter representative cores from cv. Ailsa Craig (AC), *sitiens,* and *sitiens* sprayed with either 100 mM ABA (ABA) or 5 mM L-NAME at 24 h following inoculation with *B. cinerea.* **(B)** Quantification of DAB staining in 12 cores from each treatment specified by in **(A)**. Mean staining as indicated by Image Quant (±SE) are given. Significant differences at $P < 0.05$ are indicated.

(Robert-Seilaniantz et al., 2011) and we have demonstrated a link between NO and ethylene production in *B. cinerea*-challenged *Arabidopsis* (Mur et al., 2012). We now sought to employ our on-line, *in planta* measurement platforms based on QCL detection and photoacoustic laser detection (Mur et al., 2011) to develop our understanding of the pathogenic interaction. NO production had already been reported in tomato cultures in response to *B. cinerea* and correlated with ROS generation, PCD and activity of the *S*-nitrosoglutathione modulating enzyme, *S*-nitrosoglutathione reductase (GSNOR; Pietrowska et al., 2015). Also, use of SNP has been shown to increase resistance to *B. cinerea* via mechanisms that, at least in part, were influences by MAPKinases (Zheng et al., 2014).

Our initial experiments demonstrated very rapid production (within 30 min) of NO following challenge with *B. cinerea* (**Figure 1A**). These rapid induction kinetics are suggestive of responsiveness to PAMPs as described for chitosan from *Fusarium eumartii* (Terrile et al., 2015). Our use of L-NAME; (leaving aside doubts regarding its mechanism of action; Gupta et al., 2011) which may involve targeting NR (Rasul et al., 2012) suppressed NO production and lesion development establishing NO as a source of resistance in this pathosystem (**Figure 2**). How directly NO contributes to *B. cinerea* resistance needs to be clearly determined as L-NAME treated plants also displayed reduced Et production; an established mediator of defense against *B. cinerea* (**Figure 2F**).

In considering how defense hormones act, many workers focus on how these act either singly or via interaction to increase resistance to a given pathogen or pathogens with a given infection strategy (Robert-Seilaniantz et al., 2007, 2011). Equally, ABA signaling has been shown to be "hijacked" by a range of pathogens to increase virulence (Grant et al., 2013). Delivery of effectors is a key feature of pathogen-focused suppression of host defenses and some effectors appear to target ABA signaling. The AvrB,AvrC effector domain from *Xanthomonas campestris* pv. *campestris*-induced *NCED5*, encoding a key enzyme of ABA biosynthesis, and increased ABA was important in disease development by this pathogen (Ho et al., 2013). There is some evidence that AvrPtoB can fulfill a similar role (de Torres-Zabala et al., 2007). Over-expression of AvrPtoB (also known as HopAB2) in transgenic plants induced the ABA biosynthetic gene *NCED* and ABA signaling focusing on *abscisic acid insensitive 1* (*ABI1*) a protein phosphatases type 2C (PP2C). Alternatively, the *Pseudomonas syringae* effector

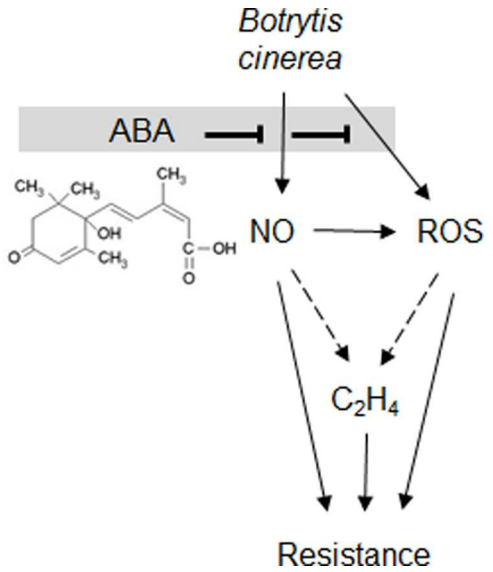

FIGURE 7 | Abscisic acid suppresses *B. cinerea* induced NO, H_2O_2 and ethylene production. Based on our observations and the literature both NO and H_2O_2 are suppressed by ABA (chemical structure shown). This will impact on the hypothesized downstream elicitation of ethylene production (shown by dashed arrows) an established source of resistance against *B. cinerea*. Thus, the relative rates of ABA production by *B. cinerea* would represent an important virulence factor.

HopAM1 enhances virulence via manipulation of sensitivity to ABA rather than initiating ABA biosynthesis (Goel et al., 2008). Moving beyond bacterial delivery of effectors, some fungal pathogen encode ABA biosynthetic genes and have been shown to synthesize ABA including *Cercopsora, Fusarium, Rhizoctonia* (reviewed by Cao et al., 2011), and of particular relevance to this current study, *B. cinerea* (Gong et al., 2014). Considering the mechanics of ABA promoted susceptibly, there is evidence that this causes reduced alterations in cell wall characteristics, ROS generation and callose deposition as well as defense gene expression (Asselbergh et al., 2007; de Torres-Zabala et al., 2007; Cao et al., 2011). Such wide ranging impact argues for multiple targets for ABA to confer increased susceptibility and paradigms from other systems; such as the phosphorylational regulation by ABA of the ROS generating NADPH oxidase AtrbohF in stomatal regulation, may be relevant (Sirichandra et al., 2009).

In order to provide a wider understanding of possible targets of ABA impacts on host responses we tested the possible interaction of ABA on NO effects. In undertaking this investigation we noted those few studies where ABA increases resistance (Ton and Mauch-Mani, 2004; Kaliff et al., 2007; De Vleesschauwer et al., 2010); thus as NO is key feature of both PTI and ETI (Mur et al., 2006) it was conceivable that ABA could increase NO generation. However, our analyses of the low ABA accumulating mutant *sitiens,* clearly indicated that ABA acted to suppress NO generation to promote virulence (**Figure 4**). This could implicate regulation of NR as a likely source of NO as a target for ABA manipulation. There is evidence for ABA acting via NR in *Arabidopsis* in the regulation of stomatal closure (Chen et al., 2015) and it is entirely conceivable that this is also the case in plant pathogen interactions. Thus, the regulation of NR by (for example) the phosphorylation of a conserved Ser residue or binding of 14-3-3 proteins could be targeted by ABA (Lillo et al., 2004). This stated it is important to unambiguously demonstrate that NR is the major source of NO in this *B. cinerea* – tomato interaction so that further work such as the use of RNAi or reduced NR activity lines is required. In this context, it should be noted that AtNOA1 although initially erroneously identified as a NO synthase (Guo et al., 2003), still contributes to NO –mediated events during plant responses to defense (Mandal et al., 2012). Indeed, although NOA1 was established as a mitochondria-located GTPase (Moreau et al., 2008) it appears to be regulating mitochondrial respiration (Heidler et al., 2011) which can be linked to NO production (Gas et al., 2009). Thus, modulation of NOA1 or mitochondrial function by ABA could be a target for ABA action. Indeed, ABA impact on certain mitochondrial GTPase and kinases (for example) have been noted (Zhao et al., 2015; Manara et al., 2016).

Increased resistance in ABA-deficient *sitiens* has been linked to elevated ROS production (Asselbergh et al., 2007) thus, the link between NO, ABA, and ROS was explored. Many studies have suggested that ROS generation is required to initiate NO production particularly in stomata (He et al., 2005; Lu et al., 2005; Bright et al., 2006; Shi et al., 2015). Additionally, ROS production was placed upstream of NO generation in the response of *Pisum sativum* to the PAMP, chitosan (Srivastava et al., 2009). Other authors have suggested that reduction of NO production through the use of NO scavengers or mutants resulted in increased ROS production (Tada et al., 2004; Asai et al., 2008) This could reflect a role for NO in modulating NADPH oxidase activity and this has been demonstrated by *S*-nitrosylation of AtrbohD (Asai et al., 2008; Yun et al., 2011). This could also reflect a role for NO as an ROS scavenger as suggested in early models (Beligni and Lamattina, 1999). In contrast, we demonstrated that the increase in ROS generation in response to *B. cinerea* in both *sitiens* and cv. Ailsa Craig could be reduced through the application of L-NAME (**Figure 6**). The placing of NO upstream of ROS generation was also suggested by Rasul et al. (2012) in oligogalacturonide-triggered immunity in *Arabidopsis*. Taken together, such studies would suggest that the relative positioning of ROS and NO is context specific; possibly even pathogen-interaction specific. Our placing of NO upstream of ROS could suggest that two major elicitory defense signals could be suppressed via a single target, i.e., NR-mediated NO production (**Figure 7**).

Considering our observations together, we have revealed a new node for pathogen-mediated suppression of host defenses, namely, NO generation. If NO is derived from NR activity, this would implicate either NR activity itself and/or N_3^-/N_2^- assimilation as likely targets for ABA-mediated modulation. If this proves to be case, questions such as if the amount of available N or its form (Gupta et al., 2013) could influence the efficacy of ABA impacts on defense and therefore host resistance, become relevant.

AUTHOR CONTRIBUTIONS

AS, AA, JM, and LM produced the data used in this manuscript. AS carried out the estimations of fungal virulence based on fungal DNA content, H_2O_2 measurements, infections of different tomato genotypes. AA also contributed to estimations of fungal virulence based on fungal DNA content. JM and LM undertook the measurements of NO and ethylene. NO and ethylene measurements were supervised by SC and FH. Overall project supervision and direction was provided by LM. The manuscript was written by LM, FH, and SC.

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REVIEW
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Nitric Oxide in the Offensive Strategy of Fungal and Oomycete Plant Pathogens

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In the course of evolutionary changes pathogens have developed many invasion strategies, to which the host organisms responded with a broad range of defense reactions involving endogenous signaling molecules, such as nitric oxide (NO). There is evidence that pathogenic microorganisms, including two most important groups of eukaryotic plant pathogens, also acquired the ability to synthesize NO *via* non-unequivocally defined oxidative and/or reductive routes. Although the both kingdoms *Chromista* and Fungi are remarkably diverse, the experimental data clearly indicate that pathogen-derived NO is an important regulatory molecule controlling not only developmental processes, but also pathogen virulence and its survival in the host. An active control of mitigation or aggravation of nitrosative stress within host cells seems to be a key determinant for the successful invasion of plant pathogens representing different lifestyles and an effective mode of dispersion in various environmental niches.

Keywords: pathogen-derived nitric oxide, nitrosative stress resistance, defense response, biotrophic pathogens, necrotrophic pathogens

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INTRODUCTION

During plant–pathogen interactions a decisive role is played by the rate and intensity of reactions induced by both adversaries. In the initiation, coordination and transfer of information on the appearing threat a particularly significant role is exerted by endogenous signaling molecules, such as nitric oxide (NO). This free radical gas can diffuse rapidly through biological membranes and it is capable of acting as a transient, local, intra-, and intercellular signal within species from every biological system.

The signaling function of NO has been shown during both highly conserved pathogen-associated molecular pattern (PAMP) triggered immunity (PTI) and in a highly specific effector-triggered immunity (ETI), often accompanied by the hypersensitive response (HR) at the site of attempted host colonization (e.g., Mur et al., 2006; Floryszak-Wieczorek et al., 2007; Asai and Yoshioka, 2009; Schlicht and Kombrink, 2013). Importantly, the generation and potential functions of NO during these interactions have so far been analyzed solely from the point of view of the host plant. Many years ago Mur et al. (2006) highlighted that the role of NO within the pathogen was too often ignored when considering plant-pathogen interactions.

The plant–pathogen system is dynamic and in the course of evolutionary changes pathogens have developed numerous invasion strategies, to which the host organism has responded with an extensive range of defense traits known as "fight for their lives". It is known from the scarce studies that also certain pathogenic microorganisms are capable of synthesizing NO, although the role of

NO in this systematically heterogeneous group of organisms has not been specified. Definitely the greatest amount of experimental data, both in terms of the sources of synthesis and the potential function of NO in the host-pathogen interactions, has been supplied by studies on model bacterial and fungal human pathogens.

Since pathogens may use NO to its own benefit and concurrently, NO may prime the host organisms and activate their defense, this review focuses on the mode of NO action in two most important groups of eukaryotic plant pathogens, i.e., fungi and oomycetes.

THE ORIGIN OF NO IN FUNGAL AND OOMYCETE PLANT PATHOGENS

Pathogens are able to produce NO; however, the origin of NO seems to be as unclear as in plants (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2014). In general, the biosynthetic pathways of NO in fungal and oomycete phytopathogens can be classified as either oxidative or reductive in operation.

The oxidative route involves NOS-like activity, which was evidenced in various groups of fungi. A pharmacological approach using mammalian NOS inhibitors, i.e., L-NAME and/or 1-[2-(Trifluoromethyl)phenyl]imidazole (TRIM), revealed a limited NO production including phytopathogenic fungi *Macrophomina phaseolina, Blumeria graminis,* and *Colletotrichum coccodes,* the mycoparasitic fungus *Coniothyrium minitans* and the aquatic fungus *Blastocladiella emersonii* (Wang and Higgins, 2005; Prats et al., 2008; Vieira et al., 2009; Li et al., 2010; Sarkar et al., 2014). Moreover, the NOS-like activity has been confirmed by measuring citrulline formation from ^{3}H-labeled arginine in the endophytic and pathogenic fungus *Neurospora crassa*, the non-pathogenic Mucorales *Phycomyces blakesleeanus* and *C. minitans* (Ninnemann and Maier, 1996; Maier et al., 2001; Li et al., 2010). Importantly, some representatives of ascomycetes and zygomycetes possess tetrahydrobiopterin, a typical mammalian NOS cofactor essential for NO synthesis (Maier and Ninnemann, 1995). Additional experiments evidenced that the catalytic activity of the fungal NOS-like enzyme depends on NADPH and involves calcium ions (Vieira et al., 2009).

Based on genome analysis NOS-like sequences were found in genomes of fungal species from the genus *Aspergillus* (*A. flavus*, *A. oryzae*, and *A. niger*) as well as *Glomerella graminicola*, the teleomorph stage of the anthracnose pathogen *Colletotrichum graminicola* (Turrion-Gomez and Benito, 2011; Sarkar et al., 2014). More recently, NOS-like protein with conserved amino acid sequences was found in the genome of *M. phaseolina*, a necrotrophic fungus causing charcoal rot disease (Sarkar et al., 2014). What is more, multiple alignments of NOS sequences followed by motif enrichment analysis have generated two motifs, one in the oxygenase domain and the other in the flavodoxin/NO synthase domain presented in separate open reading frames (ORFs). As indicated by Sarkar et al. (2014), these motifs are conserved among the five necrotrophic plant pathogens including *M. phaseolina*, *Neofusicoccum parvum*, *Colletotrichum gloeosporioides*, *G. graminicola,* and *A. oryzae*.

Available data indicate that NOS-like activity in fungi could be greatly varied. For example, in mycelia of *P. blakesleeanus* it is 10 pmol/mg/min (Ninnemann and Maier, 1996) and in fruiting bodies of white-rot basidiomycete *Flammulina velutipes* it amounts to 500 pmol/mg/min (Song et al., 2000). In *C. minitans* the highest level of NOS-like activity, amounting to 20 pmol/min/mg, was recorded during conidiation (Li et al., 2010), suggesting that this route of NO synthesis is closely regulated depending on the species, developmental stage and environmental conditions.

Evidence for the existence of an nitrate redctase (NR) dependent pathway of NO biosynthesis in *A. nidulans* was recently provided by Marcos et al. (2016). Although this reductive route was found to be functional during growth in both liquid and solid media, NR-dependent NO production was more abundant on solid media, where reproductive development occurred.

Fungi are able to produce NO as a result of denitrification processes, catalyzed by three enzymes: nitrate reductase (Nar), nitrite reductase (Nir), and NO reductase (Nor) (Ye et al., 1994; Morozkina and Kurakov, 2007). The denitrifying system coupled with the mitochondrial electron transport chain facilitates anaerobic respiration associated with ATP synthesis under hypoxia conditions. Nitrite reductases located in the intermembrane space of the fungal mitochondria have been shown to reduce NO_2^- to NO in a NADP-dependent manner (Röszer, 2012). A dissimilatory nitrite reductase was found in phytopathogens *Fusarium oxysporum* and *Cylindrocarpon tonkinense* as well as in a potentially pathogenic for humans yeast *Fellomyces fuzhouensis* (Abraham et al., 1993; Kobayashi and Shoun, 1995; Kobayashi et al., 1996; Uchimura et al., 2002). Moreover, using combination of the membrane inlet mass spectrometry (MIMS) and the restriction capillary inlet mass spectrometry (RIMS) techniques a nitrite-induced NO production has been demonstrated from cultures of plant pathogenic fungi *Botrytis* and *Fusarium* sp. and the oomycete *Pythium* (Conrath et al., 2004)

Interestingly, NO synthesis in the rice blast fungus *Magnaporthe oryzae* was associated neither with nitrite-dependent nor with the arginine-dependent pathway (Samalova et al., 2013). By creating mutants with a single and double knockout of genes potentially involved in NO synthesis, including NOS-like genes (*NOL2* and *NOL3*), nitrate (*NIA1*) and nitrite reductase (*NII1)*, the authors revealed that NO is not generated by the candidate proteins. A mammalian NOS inhibitor, L-NAME applied to a necrotroph *Botrytis cinerea* growing on a medium and *in planta* did not limit NO production either (Turrion-Gomez and Benito, 2011). The pathogen was also incubated with NO_3^- to identify the source of NO production *via* nitrate reductase, but NO signal was not detected implying that a physiological and genetic system other than NOS and NR is responsible for NO production in this plant pathogen (Turrion-Gomez and Benito, 2011). A plausible explanation for this elusive route of NO synthesis could involve an unidentified complex system of a fungus-type enzyme catalyzing NO production.

As suggested by Röszer (2012), the oxidative route of NO formation might be dominant in pathogen units under aerobic conditions and localized in the cytoplasm, whereas enzymes responsible for NO production under hypoxia or anoxia could involve mitochondrial cytochrome-c oxidase and mitochondrial nitrite reductase.

NO AS A DEVELOPMENTAL SIGNAL IN FUNGAL AND OOMYCETE PATHOGENS

Recent studies have indicated that NO may play an important role in signaling networks in fungi. Based on the pharmacological approach (including also non-pathogenic fungi) it was shown that NO participates in the development of various fungal structures including sporangiophores, conidia, cleistothecia, pycnidia and appresoria (Maier et al., 2001; Wang and Higgins, 2005; Gong et al., 2007; Prats et al., 2008; Baidya et al., 2011). However, the intensity of NO generation and its location is strictly dependent on the developmental stage of the pathogen. Bio-imaging with fluorochrome DAF-FM DA showed NO presence in *C. coccodes* conidia, germ tubes, and immature appressoria, but the strongest NO-dependent fluorescence was observed in the cells with reduced cytoplasm in the conidium with a mature appressorium (Wang and Higgins, 2005). Also in the *B. cinerea* system the production of NO was detected in all fungal developmental stages, starting from spores up to mature mycelium (van Baarlen et al., 2004; Turrion-Gomez and Benito, 2011). A strong NO signal was found in young pycnidia of *C. minitans* and in the undifferentiated tissue of pycnidial primordia; in turn, weak fluorescence signals were observed in growing hyphal tips or hyphae, where pycnidia or primordia did not develop (Gong et al., 2007).

In general, both sexual and asexual reproduction in fungi was documented to be dependent on NO generation. What is more, NO could control a threshold to switch developmental phases. It was found that a NO-releasing compound (DETA NONOate) reduced asexual development in *A. nidulans* which has a limited, but significant, phytopathogenic potential (Dean and Timberlake, 1989). In turn, formation of sexual structures was increased after NO supplementation in several fungal species, including species from the genera *Aspergillus* and *Neurospora*, as well as the species *F. velutipes* (Song et al., 2000; Baidya et al., 2011). Alterations in *A. nidulans* conidiation induced by exogenous NO may be due to NO impairing the transcriptional activation of structural sporulation specific genes (Chiuchetta and Castro-Prado, 2005). NO levels influenced the balance between conidiation and sexual reproduction, since an artificial strong elevation of NO levels reduced conidiation and induced the formation of cleistothecia. As it was found by Marcos et al. (2016), different NO levels affected the expression of the regulator of sexual development *nsdD* and the regulator of conidiation *brlA*. Baidya et al. (2011) also showed that NO is involved in the switch of developmental phases. Deletion of *fhbA*, a gene encoding flavohaemoglobin (Fhb) protein involved in the reduction and detoxification of NO, resulted in increased Hülle cell production which nurse the young fruiting body during development (Baidya et al., 2011). This result suggests that *fhbA* is likely to suppress sexual development under stressful conditions accompanied by reactive nitrogen species (RNS) overproduction (Dyer and O'Gorman, 2012).

Nitric oxide also controls the development of sporangiophores in the zygomycete *P. blakesleeanus* (Maier et al., 2001). Exposure to light signals activated asexual reproduction concomitant with NO emission within developing cells of the fungus. Exogenous NO was able to mimic the light effect on sporangiophore formation indicating that NO could function as a light sensor molecule during light-mediated sporulation. The photoconidiation process in ascomycete *N. crassa* is also dependent on NO (Ninnemann and Maier, 1996). However, NO donor inhibited light-stimulated conidiation in *N. crassa,* whereas specific inhibitors of NOS activity enhanced conidiation in darkness and in the light. The role of NO in fungal physiology is related to spore germination as well. NO synthesis was found during germination of *C. coccodes* spores. Since NO trapping accelerated germination and exogenous NO delayed this phenomenon the authors suggested that a specific NO threshold could control the time of exit from spore dormancy (Wang and Higgins, 2005).

The sporulation of the blastocladiomycete *B. emersonii* was accompanied not only by an increased level of NO and NO-derived compounds but also by the expression of genes coding for guanylyl cyclase and cGMP phosphodiesterase. Using the pharmacological approach Vieira et al. (2009) demonstrated that the Ca^{2+}-NO-cGMP signaling pathway facilitated control of zoospore biogenesis in the aquatic saprophytic fungus. Further support for the signaling role of NO during fungal development has been provided by Gong et al. (2007). The plant pathogen *Cryphonectria parasitica* failed to produce pycnidia in response to both L-NAME and the cGMP blocker (6-anilinoquinoline-5, 8-quinone), suggesting that the NO-mediated signal for conidiation may be common to multiple fungal genera, which produce pycnidia (Gong et al., 2007). Importantly, the dynamics of NO synthesis was closely related to changes in cGMP levels during pycnidial development.

NO OFFENSIVE AND NECROTROPHIC PATHOGENS

Nitric oxide was found to be produced by various plant necrotrophs, including *B. cinerea*, *A. nidulans, M. phaseolina*, *F. oxysporum*, and *C. coccodes* (Conrath et al., 2004; Wang and Higgins, 2005; Floryszak-Wieczorek et al., 2007; Turrion-Gomez and Benito, 2011; Sarkar et al., 2014). Based on a model necrotroph *B. cinerea* NO generation was detected during both saprophytic growth and *in planta* (Conrath et al., 2004; Floryszak-Wieczorek et al., 2007). Based on DAF2-DA fluorochrome, the presence of NO was found in hyphae and spores of *B. cinerea* growing on a solid medium (Floryszak-Wieczorek et al., 2007). What is noteworthy, the necrotroph contact with pelargonium leaf tissue of the susceptible genotype

resulted in the acquisition of the ability to generate much greater amounts of NO, favoring necrotic death of host cells and in consequence – disease development. The NO-dependent fluorescence surrounded germinating spores and mycelium of *B. cinerea* growing both on a complete medium and *in planta*, indicated the diffusion of NO produced inside the fungal cells (Turrion-Gomez and Benito, 2011). The observed NO spreading outside the fungal structures could have important physiological consequences in the establishment and progress of the disease, since pathogen-derived NO could reach plant cells and contribute to the hypersensitive cell death, facilitating subsequent tissue colonization (Turrion-Gomez and Benito, 2011; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2014). Noteworthy, a compound required for full virulence of *B. cinerea*, the endopolygalactouronase 1 (BcPG1) was evidenced to trigger a phosphorylation-dependent NO production in grapevine cells (Vandelle et al., 2006). Thus, NO overaccumulation *in planta* induced by pathogen or originating both from the pathogen and the host plant might accelerate the spread of infection and constitute a significant element determining success of the necrotrophic aggressor. A strong accumulation of NO in host tissue correlated with disease development was also observed in the compatible lily–*Botrytis elliptica* interaction (van Baarlen et al., 2004). In the susceptible jute–*M. phaseolina* interaction NO overproduction followed 20 dpi and coincident with NO-derived compound accumulation localized in the vascular bundle region containing invaded mycelium and micro-sclerotia (Sarkar et al., 2014). It should be noted that the necrotroph-induced NO generation in plant cells could also correlate with enhanced disease resistance. An early and only transient NO burst synchronized with ROS generation was found to positively modulate resistance in various plants under attack by necrotrophic fungal pathogens, such as *Botrytis cinerea* or *Sclerotinia sclerotiorum* (Mur et al., 2006; Floryszak-Wieczorek et al., 2007; Asai and Yoshioka, 2009; Perchepied et al., 2010).

Production of NO could also have implications in virulence of fungal pathogens *via* regulation of mycotoxin biosynthesis. For example, the Fhb gene *fhbA* influences mycotoxin biosynthesis in necrotrophic *A. nidulans* Δ*fhbA* (or Δ*fhbA* Δ*fhbB*), since deletion mutants showed a reduction of sterigmatocystin production (Baidya et al., 2011). The diminished pool of mycotoxins coincided with a decrease in the expression of *aflR*, a transcription factor necessary for the activation of the sterigmatocystin gene cluster. The NO-releasing compound application to Δ*fhbA* strains resulted in an increase of *aflR* expression levels and in the recovery of mycotoxins near the wild-type levels (Baidya et al., 2011).

NO OFFENSIVE AND HEMI-/BIOTROPHIC PATHOGENS

Pathogens with the hemibiotrophic and biotrophic life strategies are able to produce NO. This RNS was found to be generated by mycelia of *Oidium neolycopersici* (Piterková et al., 2009), *B. graminis* f. sp. *hordei* (Prats et al., 2008), *M. oryzae* (Samalova et al., 2013) and by hyphae of the oomycete *Bremia lactucae* (Sedlárová et al., 2011).

In *B. lactucae* the presence of NO was observed in the infection structures of the pathogen grown both on susceptible and resistant lettuce genotypes; however, the plant genotype determined timing of the pathogen development. A strong NO signal was detected in the tip of the germ tube and appressorium, which is a prerequisite for tissue penetration. A weaker NO signal was detected in developing primary and secondary vesicles, intracellular hyphae and in haustoria on susceptible lettuce (Sedlárová et al., 2011). The genotype with an abundant HR showed NO generation frequently localized in penetrated cells undergoing HR before the occurrence of detectable necrosis (Sedlárová et al., 2011).

Rice blast fungus *M. oryzae* produced NO during germination and early development, and was critically required for the progress of appressorium formation. Importantly, the elimination of NO produced by *M. oryzae* significantly reduced the level of infection in a compatible interaction, confirming that the pathogen-derived NO is required for successful host colonization by the hemibiotrophic pathogen (Samalova et al., 2013). Most recently, Zhang et al. (2015) revealed that the *M. oryzae* gene *MoSFA1* coding S-(hydroxymethyl)-glutathione dehydrogenase involved NO metabolism through the reduction of S-nitrosoglutathione (GSNO) contributing to full virulence in the pathogen. *MoSFA1* mutants showed attenuated virulence on rice cultivar CO-39, as well as severe reduction of conidiation and appressorium turgor pressure. Importantly, the virulence of *MoSFA* mutants on wounded rice leaves was not affected, indicating that MoSFA1 significantly contributes to virulence during penetration or the biotrophic phase of the pathogen.

Differentiation of infection structures was also found to be determined by NO in the biotroph *B. graminis* f. sp. *hordei*. NO trapping as well as blocking of NOS-like activity significantly reduced the number of appressorial lobes, which in consequence affected host cell penetration (Prats et al., 2008).

In contrast to plant-necrotroph interactions, tomato powdery mildew development was not accompanied by NO over-accumulation in host cells, although pathogen-derived NO could favor infection. NO signal was detected in *O. neolycopersici* conidia, germ tubes and appressoria developed on various genotypes of *S. lycopersicum* at 24 and 48 hpi. At later stages of pathogenesis NO was absent in spreading structures of the pathogen in susceptible tomato and only attacked cells of moderately resistant and highly resistant plants showing a strictly localized NO generation (Piterková et al., 2011).

Since the functional role of NO in living organisms may be realized directly through the involvement of NO in the post-translational modification, NO is likely to have significant implications in pathogen virulence also via the nitration/S-nitrosylation phenomenon. The analysis of a crude extract of the plant biotrophic fungus *Plasmopara halstedii* revealed the presence of nitrated proteins; however, their role remains unknown (Chaki et al., 2009).

PATHOGEN SURVIVAL UNDER NO STRESS

Colonization of the host tissues by pathogens often results in an over-production of NO and NO-derived molecules, which create boosted and pathophysiological levels of RNS, defined as nitrosative stress (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2014). Therefore, the nitrosative stress response seems to be notably important during the early stages of infection when the fungus is battling with host defenses.

Although NO over-accumulation could favor necrotroph development and/or host colonization, most of the published data rather support an adverse effect of NO on fungal growth (Schlicht and Kombrink, 2013). It remains controversial whether NO itself can kill pathogens; however, the potential antimicrobial effect of NO on pathogens has been experimentally proven. Gaseous NO inhibited mycelial growth, spore germination and sporulation of three plant pathogenic fungi, including *A. niger*, *Monilinia fructicola*, and *Penicillium italicum* under *in vitro* conditions, indicating a direct effect of exogenous NO on fungal metabolism (Lazar et al., 2008). The inhibitory effect of NO on fungal growth and spore germination of the plant pathogen *P. expansum* was related to increased levels of intracellular ROS and elevated carbonylation damage. Simultaneously, the activities of SOD and CAT as well as ATP content were diminished in response to the NO modulator (Lai et al., 2011). Moreover, the *35S::nNOS Arabidopsis* line, which contained constitutively enhanced NO levels due to the expression of the rat neuronal NOS, rendered the plants more resistant to infection by a biotrophic fungus *Golovinomyces orontii* (Schlicht and Kombrink, 2013). This transgenic *Arabidopsis* line displayed also enhanced resistance to a bacterial pathogen *P. syringae* and enhanced tolerance to salt and drought. Since the enhanced resistance was associated with SA accumulation and SA-responsive defense genes expression, the authors speculated that restriction of fungal growth was the result of diverse NO-mediated plant defense components; however, a direct effect of NO on biotroph development may not be excluded (Schlicht and Kombrink, 2013).

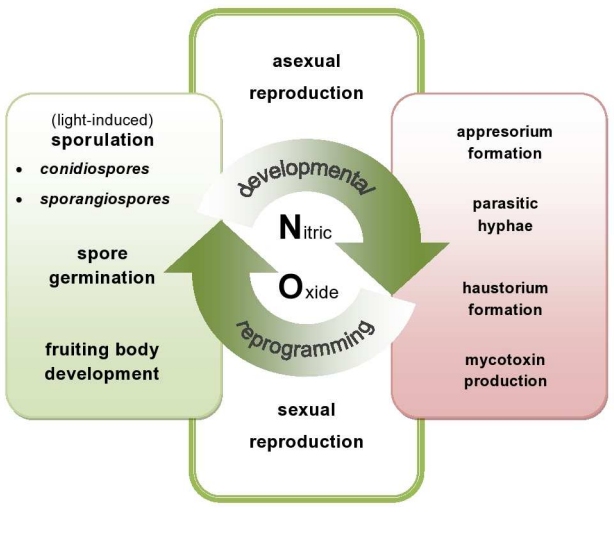

FIGURE 1 | Nitric oxide (NO)-mediated events in plant pathogenic fungi and oomycete. NO might be directly or indirectly involved in the switch between developmental phases of the phytopathogens. On the one hand, endogenously produced NO participates in various developmental processes including sporulation, germination and fruiting bodies formation (green area – NO in developmental events). On the other hand, NO may be responsible for production of toxins and differentiation of various infection structures of the pathogens (red area – NO in offensive strategy).

Phytopathogens cope both with innate NO and with plant-derived NO. In response to these huge amounts of NO pathogenic microorganisms have evolved constitutive and inducible mechanisms to prevent the adverse effects of NO, helping them to survive during the contact with host cells (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2014). Thus, the pathogen metabolic equipment to counteract nitrosative stress might be implicated in its virulence. Among inducible mechanisms, the detoxification of NO governed by the evolutionarily ancient Fhb is the best described for model fungi and human fungal pathogens (Forrester and Foster, 2012). Genes coding Fhb-like proteins have also been identified in fungal phytopathogens, including *B. cinerea*, *Cladosporium fulvum*, *F. oxysporum, Gibberella zeae*, and *N. crassa* (Boccara et al., 2005); however, the *in vivo* ability to detoxify NO via NO-dioxygenase activity has only been confirmed for *B. cinerea*. Unlike human pathogens, deletion of *Bcfhg1* did not affect pathogenicity of *B. cinerea* in relation to three different host species such as *S. lycopersicum*, *A. thaliana*, and *P. vulgaris* (Turrion-Gomez et al., 2010).

Another system contributing to nitrosative stress resistance employs *S*-nitrosoglutathione reductase (GSNOR). This GSH-dependent bi-functional enzyme is able to reduce GSNO to form GSSG plus NH_3, as well as detoxify formaldehyde (Tillmann et al., 2015). In plants, GSNOR modulates the extent of cellular *S*-nitrosothiol (SNO) formation following nitrosative stress and is required for disease resistance (Feechan et al., 2005; Yu et al., 2012). As it was mentioned before, in *M. oryze* a *MoSFA1* coding *S*-(hydroxymethyl)glutathione dehydrogenase belonging to a class III alcohol dehydrogenase was proven to be involved in NO metabolism *via* GSNO reduction (Zhang et al., 2015). As evidenced by Fernandez and Wilson (2014), GSH-recycling in the GSH-dependent antioxidant system is critical in the colonization phase of host cells by *M. oryzae*. Moreover, loss of *MoSFA1* increases the level of SNOs creating indirectly NO over-accumulation and significantly attenuated the virulence on plant hosts (Zhang et al., 2015). It worth pointing that both enzymes counteracting nitrosative stress, i.e., Fhb and GSNOR, promoted virulence of the human fungal pathogen *Cryptococcus neoformans* (de Jesús-Berrıos et al., 2003).

An unknown strategy to remove NO or suppress its excessive accumulation has been suggested for *G. orontii*. In *Arabidopsis* leaves infected with the host-adapted powdery mildew a localized and high peak of NO formation coincided in time with appressorium formation by *G. orontii* primary hyphae. Since

the NO level rapidly declined to the background level after the initial burst, the biotrophic pathogen might actively modulate its amount by degradation/decomposition mechanisms and in consequence resume growth and colonize the whole leaf (Schlicht and Kombrink, 2013). In *Candida albicans*, a human fungal pathogen, RNS exerts fungistatic effects probably by causing damages through *S*-nitrosylation of proteins and glutathione. In response to this stress, a set of genes, including RNS detoxifying enzyme, is induced. Following RNS detoxification, *C. albicans* restored redox homeostatis and the resulted *S*-nitrosylated adducts could be repaired *via* denitrosylation, allowing the pathogen to resume growth (Brown et al., 2014).

It is worth pointing out that screening of a genomic DNA library makes it possible to identify a *ntpA* gene that conferred growth tolerance upon *A. nidulans* exposure to exogenous NO (Zhou et al., 2013). The *ntpA* disruption increased amounts of cellular SNO and provoked NO susceptibility. The gene coding a cysteine-rich 23-amino-acid peptide that reacts with NO and GSNO to generate an *S*-nitrosated peptide called inducible nitrosothionein (iNT). The NO scavenging role of iNT seems to be mediated by thioredoxin-dependent catalysis. Importantly, the authors highlighted that the ubiquitous distribution of iNT-like polypeptides constitutes a potent NO-detoxifying mechanism that is conserved among various organisms (Zhou et al., 2013).

CONCLUSION

Increasing knowledge from diverse systems indicates that NO plays a pivotal role in the immune response of plants attacked by pathogens with different lifestyle. However, despite extensive research on NO synthesis and signaling processes in plants interacting with biotic stressors, our understanding of NO in phytopathogens is very limited. What is more, a validity of the reports on the biological action of NO in pathogenic microorganisms should undergo some criticism due to the methods used for NO detection. For example, the specificity of widely used DAF-based dyes is questionable since under biological conditions the fluorescent triazole product can be formed from either nitrosative or oxidative chemistry (Bryan and Grisham, 2007). Also the specificity confirmation of NO-dependent fluorescence using the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide (cPTIO) need to be used with caution because there is evidence that it can increase fluorescence (Arita et al., 2007). Finally, the use of NOS inhibitors in looking for NOS activity in fungal and oomycete pathogens does not provide an unequivocal confirmation for the enzyme presence, since these chemicals can inhibit other biosynthetic pathways as well (Planchet and Kaiser, 2006).

Fungi and oomycete pathogens have active sources of NO and pathways of its detoxication, which defend them against NO-induced damages and ensure the vital level required for the signaling function both in the pathogen physiological state and during host tissue colonization (**Figure 1**). Although pathogens with different lifestyles vary in their sensitivity to host-generated RNS, a flexible NO metabolism could constitute a good strategy to subdue the host plant.

Recognition of pathogen-derived NO during the battle between both adversaries will help us to resolve one of the central questions in plant pathology, namely what makes a pathogen successful and what makes a plant become a vulnerable host. To this end there are many important issues which need to be addressed. Firstly, research on additional genetic resources to unravel NO biosynthesis is needed. Secondly, the identification of cellular targets, degradation pathways and the mechanism/s of signal transduction need to be elucidated within different pathogen structures. Finally, fungal and oomycete pathogens, simultaneously exposed to combinations of different stimuli within the host cells, rather than to RNS alone, could activate specific NO sensing mechanisms relevant to the successful host colonization.

AUTHOR CONTRIBUTIONS

MA-J and JF-W provided the idea, wrote the paper. All authors have read and approved the manuscript.

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Nitric Oxide and Reactive Oxygen Species Coordinately Regulate the Germination of *Puccinia striiformis* f. sp. *tritici* Urediniospores

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Nitric oxide (NO) and reactive oxygen species (ROS) function as signaling molecules in a number of critical signal transduction pathways in plants, including plant biotic interactions. In addition to the role of plant-derived NO and ROS in plant resistance, which has been well documented, pathogen-produced NO and ROS have recently emerged as important players in fungal development and pathogenesis. However, the effects of pathogenic fungi-derived NO and ROS on signaling pathways during fungal pre-infection development remain unknown. Here, using a combination of pharmacological approaches and confocal microscopy, we investigated the roles of NO and ROS during the germination of *Puccinia striiformis Westend* f. sp. *tritici* (*Pst*) the wheat stripe rust pathogen. Both NO and ROS have a crucial role in uredinial germination. The scavengers of NO and ROS delayed spore germination and decreased the lengths of germ tubes. A similar phenotype was produced after treatment with the promoter. However, the spores germinated and grew normally when the levels of NO and ROS were simultaneously elevated by the application of a promoter of NO and a donor of ROS. Confocal laser microscopy indicated that both NO and ROS preferentially localized at the germ pores and apexes of growing germ tubes when the ROS/NO ratio in the spores was maintained in a specific range. We concluded that both NO and ROS are critical signaling molecules in the pre-infection development of *Pst* and that the polar growth of the germ tube is coordinately regulated by NO and ROS.

Keywords: NO, ROS, *Pst*

Abbreviations: CLSM, confocal laser scanning microscopy; c-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, 4-amino-5-(*N*-methylamino)-2, 7-difluorofluorescein diacetate; H_2DCF DA, 2′, 7′-dichlorodihydrofluorescein diacetate; DAF-FM T, 4-amino-5-(*N*-methylamino)-2, 7-difluorofluorescein triazole; DCF, dichlorofluorescein; DPI, diphenyliodonium iodide; HPG, hours post germination; L-NAME, *N*-nitro-L-arginine methyl ester; MFI, mean fluorescence intensity; MGL, mean germ tube lengths; MGR, mean germination rate; NADPH, nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; NO, nitric oxide; NOS, nitric oxide synthase; *Pst*, *Puccinia striiformis* f. sp. *Tritici;* RNS, reactive nitrogen species; ROS, reactive oxygen species.

INTRODUCTION

Two important types of free radicals, NO and ROS, are crucial signaling molecules involved in a number of signal transduction pathways. The roles of NO and ROS in mammals have been studied for many years. They are crucial messengers in the immune, nervous, and cardiovascular systems (Palmer et al., 1987). In plants, they are involved in several physiological processes, including seed germination and lateral leaf and root development, and have been implicated in both abiotic and biotic stress responses (Besson-Bard et al., 2008; Wilson et al., 2008; Swanson and Gilroy, 2010). Indeed, there is considerable evidence that plant-derived NO and ROS are important in initiating plant responses to pathogens or elicitors (Brisson et al., 1994; Levine et al., 1994; Delledonne et al., 1998; Chaki et al., 2009).

Evidence is also emerging that NO and ROS are important regulatory molecules in microbe, including plant pathogens. It has been found that ROS is involved in the germination and germ tube growth of conidia of *Cladosporium fulvum* (Lu and Higginsf, 1999) and the biofilm resistance of *Pseudomonas aeruginosa* (Elkins et al., 1999).

It was reported that pathogen-derived NO influences germination in *Colletotrichum coccodes* (Wang and Higgins, 2005), conidiation in *Coniothyrium minitans* (Gong et al., 2007) and sporangiophore development in *Phycomyces blakesleeanus* (Maier et al., 2001) and affects the formation of appressoria in the obligate biotrophic powdery mildew fungus *Blumeria graminis* (Prats et al., 2008) and pathogenicity in the rice blast fungus *Magnaporthe oryzae* (Averyanov and Lapikova, 1990).

Reactive oxygen species have been reported to be involved in fungal virulence and development (Heller and Tudzynski, 2011). There are various reports on the effects of ROS free radicals on spore germination. O_2^- and OH radicals were both detected during the germination of *Pyricularia oryzae*, and the radical scavengers superoxide dismutase (SOD), catalase and OH increased the percentage of germination (Averyanov and Lapikova, 1990). During spore germination in *Neurospora crassa*, an accumulation of catalase was observed, indirectly suggesting that H_2O_2 was generated in the process (Michan et al., 2002).

Wheat stripe rust, caused by *Pst*, is one of the most important diseases of wheat and can cause significant loss to wheat yield and grain quality (Chen, 2005). Although *Pst* is a macrocyclic rust pathogen, its propagation and spread occur primarily by means of urediniospores, which are capable of germination and infection under suitable environmental conditions immediately after release (Chen et al., 2014). Following the initiation of germination, the cytoplasm of a urediniospore moves into the germ tube until it reaches a stoma.

To our knowledge, no study has suggested a role for NO and ROS during urediniospore germination. Hence, CLSM was used to visualize NO and ROS generated by urediniospores *in vivo* and the role of NO and ROS in *Pst* development was investigated. We identify a regulatory role for NO and ROS during the germination of urediniospores and the apical growth of germ tubes in *Pst* using specific probes and the donor/promoter and scavengers of NO and ROS. The scavengers of NO and ROS delayed germination and decreased germ tube length. Moreover, a promoter of NO and a donor of ROS could also delay germination and decrease the germ tube length. Further study determined that the spores germinate when the ROS/NO ratio is maintained within a specific range and that NO and ROS primarily exist in the apex of the germ tube, suggesting that both NO and ROS are involved in apical germ tube growth.

MATERIALS AND METHODS

Pathogen and Reagents

Fresh urediniospores of *Pst* pathotype CYR31 used in this study were provided by the Institute of Plant Pathology, Northwest A&F University. The specific NO scavenger c-PTIO (Balcerczyk et al., 2005), the substrate of NO synthesis L-Arg (Bonilla et al., 2004; for clarity, L-Arg is elsewhere called "the promoter of NO"), the ROS donor triphosphopyridine nucleotide (NADPH), the NADPH scavenger DPI, NO-specific probe 4-amino-5-(*N*-methylamino)-2,7-difluorofluorescein diacetate (DAF-FM DA) and the ROS-specific probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF DA) were used in this study. All reagents used in this study were obtained from Sigma–Aldrich, USA.

Determination of Appropriate Reagent Concentrations

Different reagents at different concentrations (c-PTIO at 0, 50, 100, 150 μM; L-Arg at 0, 2, 3, 4 mM; DPI at 0, 10, 20, 30 μM and NADPH at 0, 2.5, 3.5, 4.5 mM) were tested in order to find the appropriate concentrations for use in the experiments.

Different probes at different concentrations (DAF-FM DA at 1, 2, 5, 10, and 15 μM and H_2DCF DA at 20, 30, 50, 70, and 100 mM) were tested in order to find the appropriate concentrations to detect the generation of NO and ROS in the study.

Finally, concentration of c-PTIO at 100 μM, L-Arg at 2 mM, DPI at 20 μM, NADPH at 2.5 mM, DAF-FM DA at 10 μM and H_2DCF DA at 50 mM were selected.

Effects of NO and ROS on Urediniospore Germination and Germ Tube Growth

Fresh urediniospores (0.6 mg) were added to 10 ml distilled water, 10 ml 100 μM c-PTIO, 10 ml 2 mM L-Arg, 10 ml 20 μM DPI, 10 ml 2.5 mM NADPH, 5 ml 20 μM DPI+5 ml 100 μM c-PTIO (c-PTIO +DPI), 5 ml 2 mM L-Arg+5 ml 2.5 mM NADPH (L-Arg +NADPH), 5 ml 20 μM DPI+5 ml 2 mM L-Arg (L-Arg +DPI) or 5 ml 2.5 mM NADPH+5 ml 100 μM c-PTIO (c-PTIO +NADPH) and allowed to germinate at 9°C in darkness.

Evidence for, and Localization of, Endogenous NO and ROS in Germinating *Pst* Urediniospores

The specific fluorescence probes-H_2DCF DA and DAF-FM DA were used to detect ROS and NO. In the presence of ROS and NO, H_2DCF DA and DAF-FM DA were converted to fluorescent

DCF and DAF-FM triazole (DAF-FM T), which could be detected separately by bright green fluorescence in CLSM.

Germinating urediniospores treated as described above (see Effects of NO and ROS on Urediniospore Germination and Germ Tube Growth) were collected at different time points and mixed with 10 μM DAF-FM DA or 50 mM H_2DCF DA prior to incubation in darkness at 25°C for 30 min. They were then immersed twice for 10 min in Tris-HCl buffer (50 mM, pH 7.4) to remove excess dye. A distilled water control without DAF-FM DA or H_2DCF DA staining and with the same other procedures was treated as CK to detect the autofluorescence of germinating urediniospores. Then, the urediniospores were placed on slides to detect the generation of NO and ROS by CLSM (LSM 510 META, Zeiss Corporation, Germany). Fluorescence was detected at an excitation frequency of 488 nm, and emission was filtered using a 515–530 nm barrier filter. NO and ROS were detected with the same parameters (gain, magnification and so on) across all treatments. Images were recorded after DAF-FM DA and H_2DCF DA staining, and observations of the CK enabled discrimination between autofluorescence (also excited by the argon laser) and fluorescence due to NO and ROS generation. The MFI values were measured in different locations of the urediniospores and germ tubes using Image Pro Plus software (IPP software, USA).

Rates of Germination and Germ Tube Lengths

Germinating urediniospores were collected at different time points and placed on slides to count the numbers of germinated urediniospores and to measure the lengths of germ tubes (a germ tube length greater than one-half the spore diameter was defined as germination) using an Olympus BX51 microscope (Olympus Corporation, Japan). The germination rate was expressed as a percentage based on 100 urediniospores.

Statistical Analysis

One hundred urediniospores were analyzed in every treatment at random, and all experiments were performed at least three times. Only representative images are shown in the paper. Differences in germination rates, germ tube lengths and mean pixel intensity among the treatments were analyzed by one-way ANOVA with the least significant differences (LSD) test at 0.05 probability level. All statistical tests were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Promoter and Scavenger of NO and ROS Affect Spore Germination

After treatment with c-PTIO and DPI, spore germination was significantly suppressed and delayed in germination time compared with spores treated with distilled water (**Figures 1** and **2**), as measured by MGL and MGR.

Urediniospores began to germinate after 1.5 h in distilled water, and the MGR and MGL were 8% and 21.3 μm, respectively; they increased over time, especially at 4–6 hpg, and peaked at 6 hpg (78.2% and 194.6 μm). By contrast, the urediniospores maintained dormancy for 1–1.5 hpg when treated with DPI and c-PTIO at 2 hpg. The MGR values were 7.7 and 8.3%, and the MGL values were 20.8 and 23.4 μm, respectively. Although the MGR and MGL increased over time, there were significant differences compared with the distilled water control. Thus ROS and NO had important roles in the germination of spores and in germ tube growth.

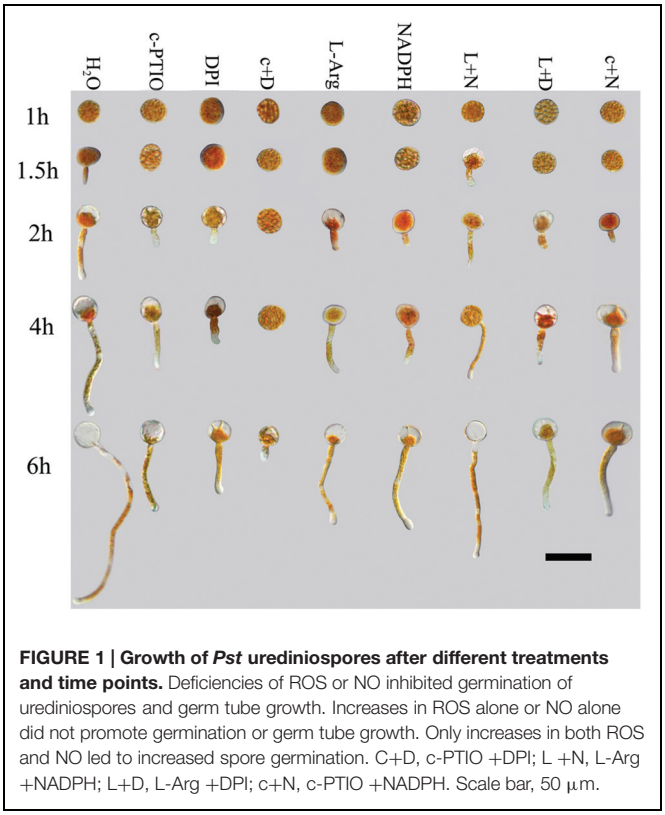

FIGURE 1 | Growth of *Pst* urediniospores after different treatments and time points. Deficiencies of ROS or NO inhibited germination of urediniospores and germ tube growth. Increases in ROS alone or NO alone did not promote germination or germ tube growth. Only increases in both ROS and NO led to increased spore germination. C+D, c-PTIO +DPI; L +N, L-Arg +NADPH; L+D, L-Arg +DPI; c+N, c-PTIO +NADPH. Scale bar, 50 μm.

After treatment with L-Arg and NADPH, urediniospore germination was also significantly suppressed (**Figures 1** and **2**). The data for MGR and MGL showed no differences between L-Arg, NADPH, c-PTIO, and DPI treatments at any time points, which suggested that an increase in ROS only or NO only could not promote spore germination or germ tube growth.

After treatment with L-Arg +DPI and c-PTIO +NADPH, the MGL and MGR were increased compared with the DPI and c-PTIO treatment (**Figures 1** and **2**). However, variance analysis showed no significant difference, indicating that excess NO or ROS did not promote spore germination or germ tube growth.

Germination was almost completely suppressed after treatment with c-PTIO +DPI (**Figures 1** and **2**), and variance analysis indicated significant differences compared with other treatments, especially distilled water.

In the presence of L-Arg +NADPH, spore germination was indistinguishable from that observed in distilled water (**Figures 1** and **2**). However, there was a significant difference between L-Arg +NADPH and distilled water at 4 hpg.

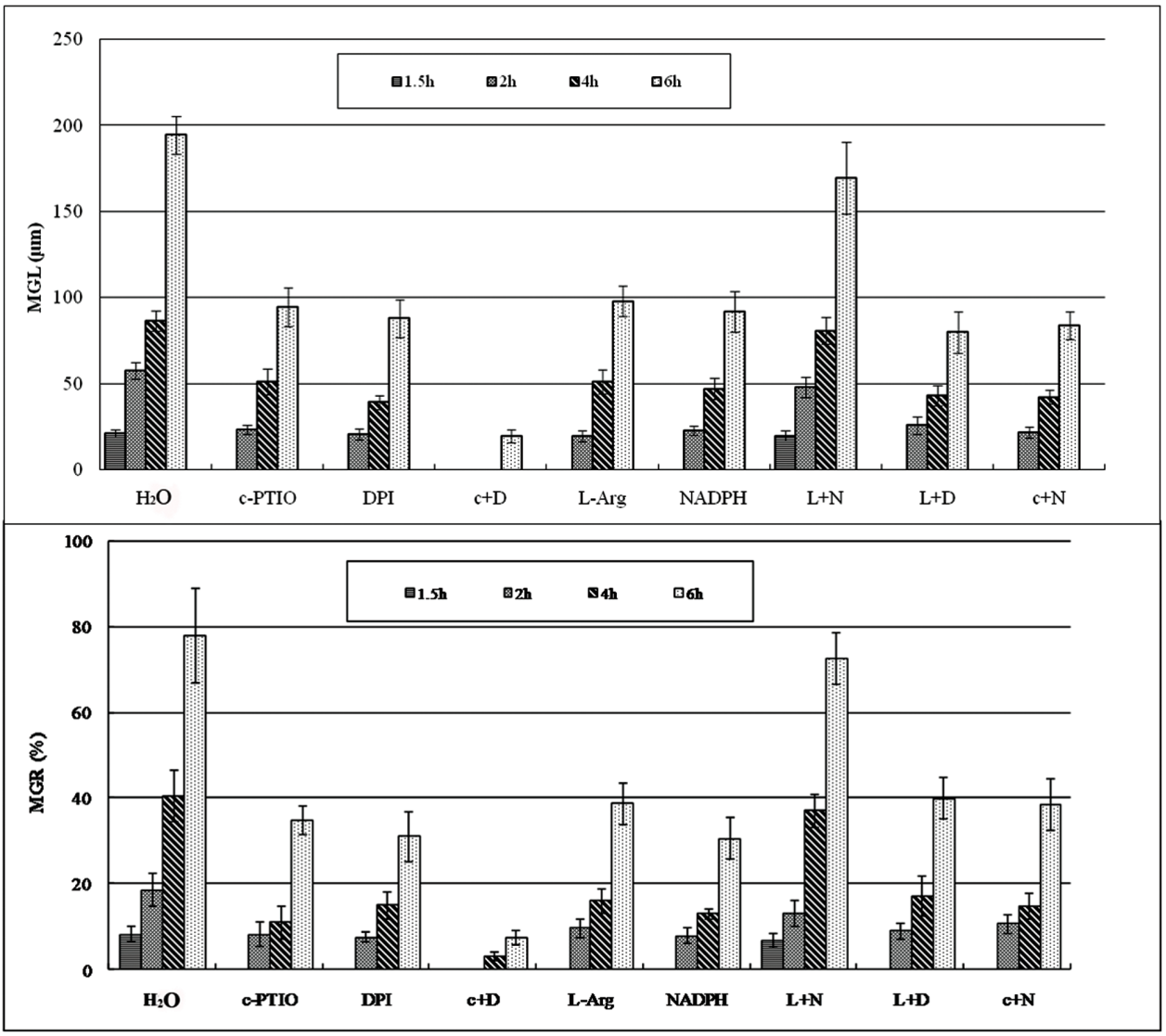

FIGURE 2 | The mean germ tube lengths and mean germination rate during *Pst* spore germination after different treatments at different time points. MGL, mean germ tube length; MGR, mean germination rate.

Thus, spore germination and fungal growth were significantly reduced when NO or ROS levels were decreased (**Figures 1** and **2**), and increases in ROS only or NO only failed to promote increases (**Figures 1** and **2**), suggesting that NO and ROS play a crucial role in spore germination and germ tube growth. This result suggested that an optimum ratio of ROS to NO possibly exist during urediniospore germination in *Pst*.

The Fluorescence Intensity of Endogenous NO Generated During Urediniospore Germination Following Different Treatments

Nitric oxide-specific fluorescent probe DAF-FM DA and CLSM were used to detect NO produced during urediniospore germination. The urediniospore and the germ tube were divided into three parts (urediniospore, base of germ tube and apex of germ tube), as shown in **Figure 3**.

CSLM performed after DAF-FM DA staining revealed the generation of NO in *Pst* spores and germ tubes.

Bright fluorescence was observed after treatment with distilled water and DAF-FM DA (**Figure 4A**); faint fluorescence was observed in distilled water without DAF-FM DA staining (CK; **Figure 4B**); c-PTIO-treated samples also displayed faint and uniform fluorescence throughout the study (**Figure 4C**). Combining the results of these three treatments, it could be confirmed that the fluorescence in **Figure 4B** is the autofluorescence of urediniospore, and the bright green fluorescence in **Figure 4A** is due to NO generation and not autofluorescence.

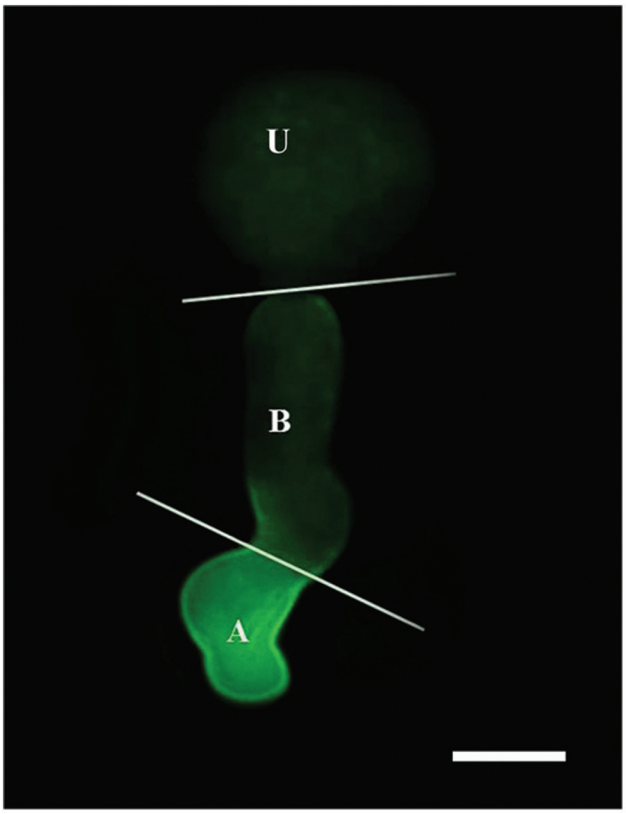

FIGURE 3 | Image of a germinating urediniospore at 2 hpg when stained with DAF-FM DA. Intense green fluorescence indicates the presence of NO in the apex of the germ tube. U, urediniospore; B, base of germ tube; A, apex of germ tube. Scale bar, 10 μm.

Bright green fluorescence was detected in germ pores, indicating that NO was generated around the germ pores during the urediniospore water-swelling stage at 1 hpg. However, germ tubes emerged from the germ pores at 1.5–2 hpg, and staining showed pronounced fluorescence in most short germ tubes, especially at their tips (**Figure 4A**). Further images showed intense fluorescence localized at the apices of germ tubes at 2–6 hpg, although faint fluorescence was also observed in the spores and bases of the germ tubes (**Figure 4A**). The same trends were observed after treatment with L-Arg +NADPH (**Figure 4H**), L-Arg (**Figure 4F**), and L-Arg +DPI (**Figure 4I**), although germination was delayed in the last two treatments.

After treatment with DPI or NADPH, visual inspection and staining with DAF-FM DA showed a constant faint fluorescence in the spores and germ tubes throughout the study (**Figures 4D,G**), showing that both the suppression and promotion of ROS inhibited NO.

When ROS and NO were both inhibited, the MFI of NO was decreased (**Figure 4E**), and spore germination was considerably delayed (**Figures 1** and **2**). After treatment with c-PTIO +NADPH, staining with DAF-FM DA showed faint fluorescence in the spores and germ tubes (**Figure 4J**).

Urediniospores treated with L-Arg, L-Arg +NADPH and L-Arg +DPI, when stained with DAF-FM DA, displayed a marked bright fluorescence correlated with the development of germ tubes (**Figures 4F,H,I**).

After treatment with L-Arg +DPI, the bright fluorescence at the spores and germ tubes were observed (**Figure 4I**), which suggested that although L-Arg could help to relieve ROS depression and produce more NO, it exhibited no enhancement on the elongation of germ tubes (**Figures 1** and **2**).

It was also observed that only when the ROS and NO were simultaneously added (**Figure 4H**) could the spore germinate normally, as in the distilled water treatment (**Figures 1** and **2**).

These results show that NO plays a crucial role in apical growth and that ROS is involved in the generation of NO. NO production was greatly increased by L-Arg, further suggesting a role for a NOS enzyme as the source of NO generation in the fungus.

Fluorescence Intensity of Endogenous ROS Generated During Germination After Different Treatments

Reactive oxygen species-specific fluorescent probe H_2DCF DA and CLSM were used to detect the ROS produced during urediniospore germination.

Bright green fluorescence was observed for the treatment with distilled water and H_2DCF DA (**Figure 5A**); faint fluorescence was observed in the distilled water without H_2DCF DA staining (CK; **Figure 5B**); DPI-treated samples also displayed faint and uniform fluorescence throughout the study (**Figure 5D**). Combining the results of these three treatments, it could be confirmed that the fluorescence in **Figure 5B** was the autofluorescence of urediniospores, whereas the bright green fluorescence in **Figure 5A** was due to ROS generation and not autofluorescence.

Bright green fluorescence indicated that ROS was generated around germination pores during the water-swelling stage at 1 hpg (**Figure 5A**). During the growth of the germ tubes, the characteristic of ROS generation were similar to DAF-FM DA staining (**Figure 5A**). The same trends were observed after treatment with L-Arg +NADPH (**Figure 5H**), NADPH (**Figure 5G**) and c-PTIO +NADPH (**Figure 5J**), although the germination was delayed in the last two treatments.

After treatment with the NO scavenger c-PTIO or the NO promoter L-Arg and staining with H_2DCF DA, only faint fluorescence was detected at the tips of germ tubes (**Figures 5C,F**). This result indicated that either the suppression or the promotion of NO decreased the concentration of ROS during spore germination.

Urediniospore treatment with NADPH, L-Arg +NADPH or c-PTIO +NADPH and staining with H_2DCF DA showed a markedly bright fluorescence during the germination of urediniospores (**Figures 5G,H,J**).

When the ROS and NO were both restrained, the MFI of ROS was decreased (**Figure 5E**) and spore germination was delayed (**Figures 1** and **2**) which was similar to DAF-FM DA staining.

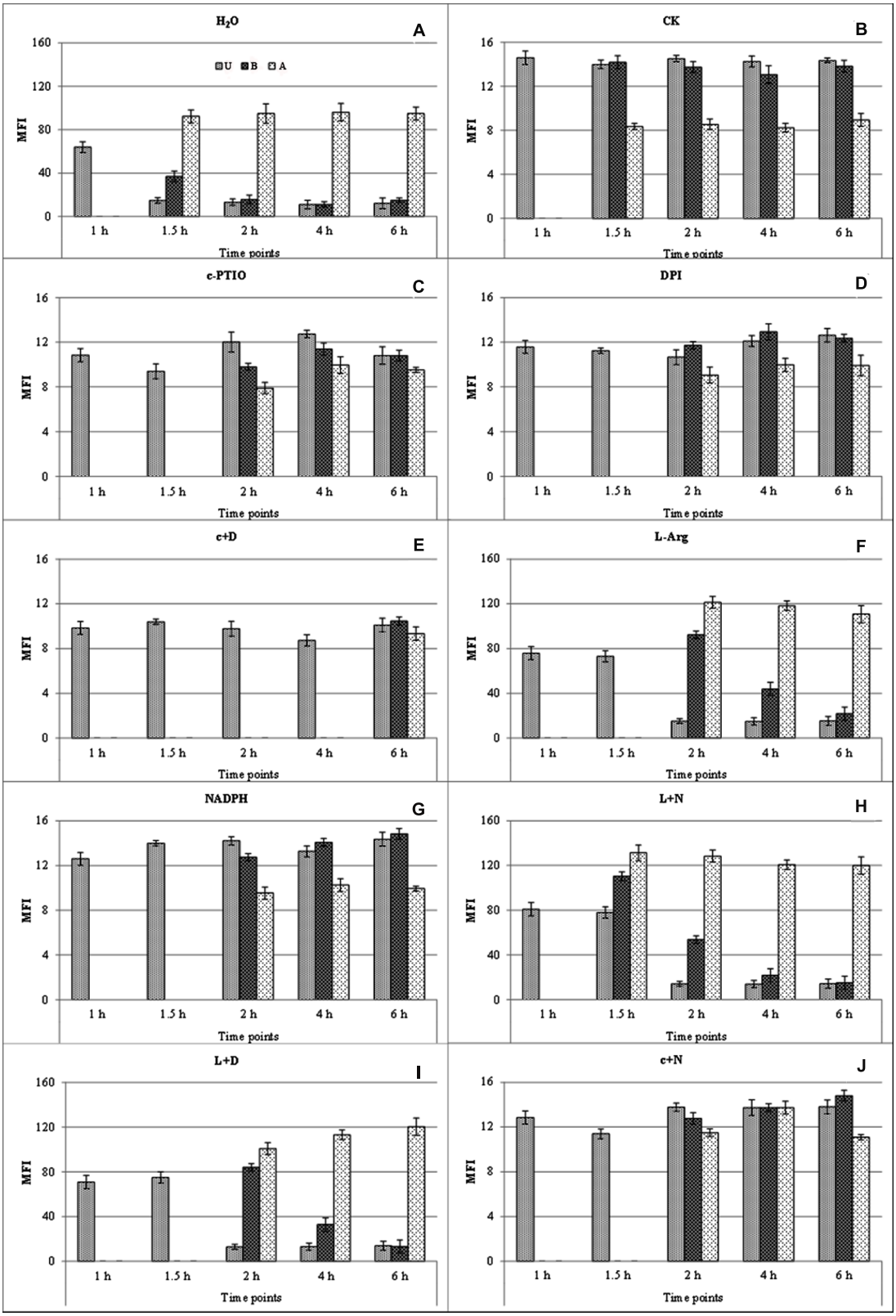

FIGURE 4 | Mean fluorescence intensity of endogenous NO generated during germination at different time points after different treatments. Bright fluorescence was observed after treatment with distilled water (H_2O) and DAF-FM DA **(A)**; faint fluorescence was observed in distilled water without DAF-FM DA staining (CK) **(B)**. U, urediniospore; B, base of germ tube; A, apex of germ tube. c+D, c-PTIO +DPI; L+N, L-Arg +NADPH; L+D, L-Arg +DPI; c+N, c-PTIO +NADPH.

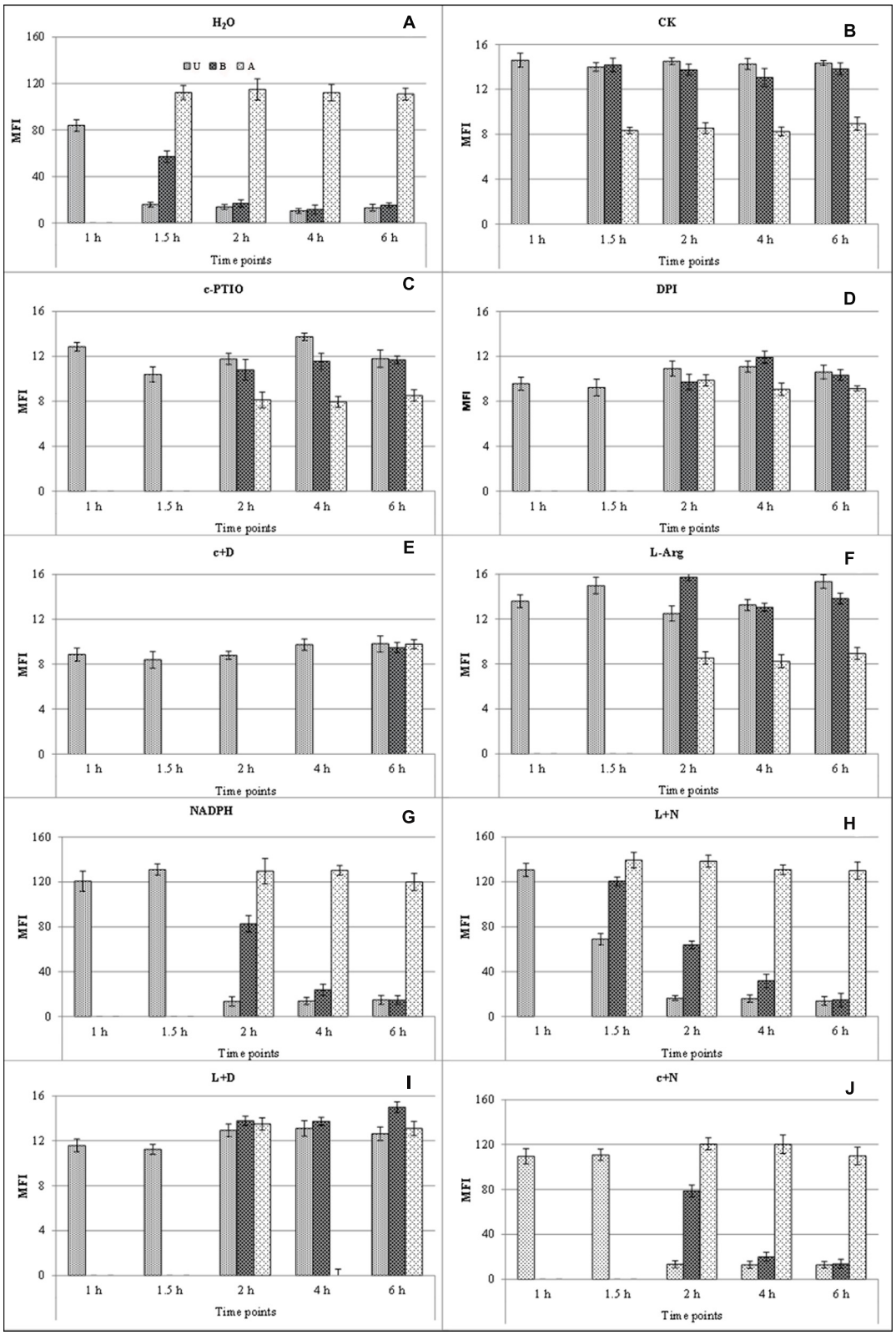

FIGURE 5 | Mean fluorescence intensity of endogenous ROS generated during germination at different time points and treatments. Bright fluorescence was observed after treatment with distilled water (H_2O) and H_2DCF DA **(A)**; faint fluorescence was observed in distilled water without H_2DCF DA staining (CK) **(B)**. U, urediniospore; B, base of germ tube; A, apex of germ tube. c+D, c-PTIO +DPI; L+N, L-Arg +NADPH; L+D, L-Arg +DPI; c+N, c-PTIO +NADPH.

After treatment with L-Arg +DPI and staining with H_2DCF DA, the urediniospore showed faint fluorescence in the spores and germ tubes (**Figure 5I**).

There was a significant difference between treatments with c-PTIO and c-PTIO +NADPH in the urediniospores at 1 hpg and in the apex of germ tubes at 2–6 hpg after H_2DCF DA staining (**Figures 5C,J**). However, the MGL were not increased (**Figures 1** and **2**), which suggested that although NADPH could help to relieve NO depression and produce more ROS, it did not enhance the elongation of germ tubes.

It was also observed that only when the ROS and NO were promoted simultaneously (**Figure 5H**), the spore could germinate normally as in the distilled water treatment (**Figures 1** and **2**).

These results show that ROS plays a crucial role in the apical growth of urediniospores and NO is involved in the generation of ROS. ROS production was greatly increased by NADPH, further suggesting a role for NADPH as the source of ROS generation in the fungus.

ROS/NO Ratio

The fluorescence intensity was directly proportional to the content of NO or ROS, so the ratio of ROS/NO fluorescence intensity could indirectly reflect the ratio of ROS/NO content in germinated urediniospores.

The MFI values of urediniospores treatment with H_2O and L-Arg +NADPH (the urediniospores after the two treatments could germinate normally) were recorded and analyzed (**Table 1**).

The ratio of ROS/NO at different locations and time points showed that spores germinated normally when the ROS/NO ratio maintained within the range of 0.9–1.6.

DISCUSSION

In eukaryotes, NO is generated from many oxynitrides such as nitrite (NO_2^-) under acid conditions (Castello et al., 2008). In addition to chemical synthesis, NO can be generated by enzymatic reactions. In animals, NO is synthesized from O_2 and L-Arg by different NO synthase (Bonilla et al., 2004) isoforms (Nowles and Moncada, 1994). Ninnemann and Maier (1996) reported NO synthase activities in fungi for the first time. NO synthase activity was detected during sporulation in *Blastocladiella emersonii* (Bonilla et al., 2004), and activity decreased significantly with the addition of L-NAME (Vieira et al., 2009). These results suggested that there was an enzymatic pathway of NO synthesis in fungi that was similar to that of mammals. In our study, endogenous NO increased sharply when the substrate of NO synthase L-Arg was added. L-Arg generates NO through the NOS catalysis pathway. Green fluorescence could still be observed after staining with the specific NO fluorescence probe, and the intensity fluorescence decreased dramatically after c-PTIO treatment. Therefore, endogenous NO might be generated by the NOS pathway during the germination of *Pst* urediniospores. It has been suggested that NO synthesis in phytopathogenic fungi is derived from an L-Arg-dependent pathway by a NOS-like system, as in the ascomycete fungi *C. coccodes* and *B. graminis* (Wang and Higgins, 2005; Prats

TABLE 1 | Reactive oxygen species/NO ratio after H_2O and L+N treatments.

Treatment	Localization	Free radicals	Time points				
			1 h	1.5 h	2 h	4 h	6 h
H_2O	U	ROS	83.8 ± 5.3	15.7 ± 2.1	13.9 ± 1.5	10.3 ± 2.7	13.0 ± 3.1
		NO	63.8 ± 4.1	14.7 ± 1.3	13.1 ± 1.3	11.0 ± 2.7	12.1 ± 3.0
		ROS/NO	1.3	1.1	1.1	0.9	1.1
	B	ROS	0	57.0 ± 5.1	16.8 ± 2.7	11.6 ± 3.0	15.3 ± 2.1
		NO	0	37.0 ± 5.2	15.7 ± 2.2	11.2 ± 1.4	15.0 ± 2.8
		ROS/NO	/	1.5	1.1	1.0	1.0
	A	ROS	0	112.2 ± 7.1	114.8 ± 8.4	112.0 ± 7.0	110.9 ± 7.1
		NO	0	92.2 ± 6.0	94.8 ± 8.4	96.0 ± 8.1	94.9 ± 6.2
		ROS/NO	/	1.2	1.2	1.2	1.2
L+N	U	ROS	130.9 ± 5.9	68.7 ± 5.0	16.5 ± 2.2	15.5 ± 3.2	14.0 ± 3.0
		NO	80.9 ± 5.9	78.0 ± 6.7	14.2 ± 1.9	14.0 ± 3.7	14.4 ± 4.0
		ROS/NO	1.6	0.9	1.2	1.4	1.0
	B	ROS	0	120.4 ± 5.2	63.9 ± 5.0	31.9 ± 3.2	14.9 ± 2.2
		NO	0	110.4 ± 4.4	53.9 ± 3.4	21.9 ± 5.9	15.2 ± 5.9
		ROS/NO	/	1.1	1.2	1.5	1.0
	A	ROS	0	139.3 ± 8.2	138.3 ± 9.5	130.7 ± 8.2	129.9 ± 9.2
		NO	0	131.3 ± 7.4	128.3 ± 5.3	120.7 ± 4.3	119.9 ± 7.7
		ROS/NO	/	1.1	1.1	1.1	1.1

The MFI values of ROS and NO of the spores treated with H_2O and L + N (spores after the two treatments could germinate normally) was collected. The ratio of ROS/NO was calculated at different locations at different time points. U, urediniospore; B, base of germ tube; A, apex of germ tube.

et al., 2008). However, fungi do not contain NOS-like sequences in their genomes, except for *Aspergillus* species and *Glomerella graminicola* (Turrion-Gomez and Benito, 2011). Genetic studies indicated that NO synthesis in *M. oryzae* was not associated with an arginine-dependent pathway, although relatively weak NOS-like sequences were present in the genome (Samalova et al., 2013). It should be feasible to validate this result by identifying the NOS in *Pst* using the available genome sequence (Zheng et al., 2013).

There is growing evidence that certain specific enzymes, such as NADPH oxidase (NOX), produce ROS to regulate cellular functions, such as immunity, cell proliferation, cell differentiation, signal transduction, and ion transport (Finkel, 2003; Foreman et al., 2003; Kwak et al., 2003; Lambeth, 2004). In this study, it was found that although spore germination was restrained after NADPH treatment, a mass of green fluorescence was still detected by the ROS-specific fluorescence probe, and the intensity of this green fluorescence decreased dramatically after DPI treatment. Thus, during urediniospore germination, endogenous ROS might be generated by the NADPH pathway.

During the study, we observe that the wax layer and the epidermal hairs on the surface of the wheat leaves would cause the gathering of ROS/NO fluorescence probes which could interfere with the accuracy of the results by CLSM. This causes the difficulty to the research of the role of NO and ROS during the germination of urediniospore. Fortunately, *Pst*, as an obligate biotroph urediniospores, can germinate and form a germ tube on the water surface which cause the feasibility of the research in the pre-infection process *in vitro* under controlled conditions.

After hydration, a urediniospore germinates and develops a germ tube that can extend along the water surface. During germination, it is believed that fungal spores undergo an initial period of isotropic expansion associated with the uptake of water. Upon the establishment of a polarity axis, a short germ tube emerges and grows by apical extension, which is a defining feature of the filamentous fungi (Harris, 2006; Riquelme, 2013). Thus, the pre-infection development of *Pst* essentially involves the transition from isotropic growth to polarized growth, and it has been proposed that polarized hyphal growth requires the establishment of polarity during spore germination and maintenance of polarity during germ tube elongation (Momany, 2002). In this study, NO and ROS preferentially localized to the spore pore and apical region of the germ tube, suggesting that they are associated with these processes. Accumulating evidence indicates that there is a correlation between ROS production by NADPH oxidase and the polarized growth of fungal cells (Glasauer and Chandel, 2013). Localized production of ROS at the growing hyphal tips was detected by NBT or H_2DCF DA staining for several fungal species, including *Epichlöe festucae*, *M. grisea,* and *Aspergillus nidulans* (Tanaka et al., 2006; Egan et al., 2007; Semighini and Harris, 2008). Further experiments performed in *M. grisea* showed that the inhibition or scavenging of ROS production by the NADPH oxidase inhibitor DPI or by the antioxidant ascorbate inhibited or impaired fungal polarized growth, which was detected as inhibition or delay of germination of the conidia and aberrant morphology of the germ tubes or appressoria (Egan et al., 2007). In the mutualistic, endophytic fungus *E. festucae*, it was demonstrated that ROS generation requires the functional assembly of a multisubunit complex composed of NoxA, a regulatory component, NoxR, and the small GTPase RacA (Takemoto et al., 2007; Tanaka et al., 2008), whereas BemA and Cdc24, well-characterized regulators of polarity in yeast, were identified as interacting with the Nox complex via NoxR (Takemoto et al., 2011). Significantly, GFP fusions of NoxR, RacA, Cdc24, and BemA preferentially localized to actively growing hyphal tips, where they functioned as an activated NADPH oxidase enzyme complex responsible for the production of ROS (Takemoto et al., 2011). These results together with our observations indicate that the NADPH oxidase-dependent production of ROS plays a conserved role in polarized hyphal growth. It is well known that the fungal cytoskeleton plays a crucial role in polarity establishment, maintenance and polar growth (Harris, 2006; Riquelme, 2013). This finding was validated in *Pst* by functional analysis of the actin gene *PsACT1* (Liu et al., 2012). A recent study revealed that ROS may regulate filamentous polarized fungal growth by remodeling the arrangement of the F-actin cytoskeleton, whereas the latrunculin-mediated depolymerization of fungal appressorial F-actin is competitively inhibited by fungal NADPH oxidases mediated by ROS (Rydera et al., 2013). Thus, we can speculate that the production of ROS by the *Pst* NADPH oxidase complex in *Pst* regulates polarized growth by reorganizing components of the cytoskeleton, such as F-actin.

In addition to ROS, we provide evidence that NO is involved in polarized growth during spore germination and subsequent germ tube growth. As with ROS, interference in NO production by application of scavenger or promoter delayed spore germination and impaired germ tube growth. A similar role has also been indicated in other fungi. For instance, the application of external NO to *C. coccodes* delayed spore germination, whereas treatment with L-Arg accelerated the germination and development of conidiospores (Wang and Higgins, 2005). Similarly, in the hemibiotrophic ascomycete *M. oryzae,* NO scavengers delayed germination and reduced lesion formation (Samalova et al., 2013). These data and our observations collectively indicate that NO may have a role in signaling in spore germination and polarized growth in fungi.

Significantly, NO seems to act in concert with ROS to control germination and germ tube growth because the elevation or reduction of NO or ROS alone has a negative effect on these processes, whereas the accumulation of high level of NO and ROS results in normal growth in *Pst*. It is likely that a balance between NO and ROS, rather than these molecules functioning alone, allows germination to proceed while ensuring that it does so only under ideal environmental conditions (Wang and Higgins, 2005).

It has been demonstrated that NO and ROS signaling pathways in plant biotic interactions are closely connected (Scheler et al., 2013). Furthermore, there is evidence showing that ROS can influence NO levels and vice versa (Moncada and Erusalimsky, 2002; Desikan et al., 2004). For example, the regulation of ROS production by NO is thought to modulate the development of the hypersensitive response (HR), a programmed cell death involved in plant defense (Yun et al., 2011; Rasul et al., 2012). NO and ROS crosstalk during fungal development may be resolved only after the characterization of all fungal NOS isomers

(Wang and Higgins, 2005). We found that upon treatment with either the promoter or scavenger of NO, ROS generation in *Pst* urediniospores was inhibited. Similarly, upon treatment with either the donor or scavenger of ROS, the generation of NO was inhibited. A critical balance of ROS and NO seems to be essential in regulating urediniospore germination and germ tube development in *Pst* and other fungi.

The generation of ROS during the interaction of fungus and its host has been repeatedly studied. There is evidence that the host produces ROS (Wang et al., 2007, 2010; Zhang et al., 2012) and NO (Romero-Puertas et al., 2004; Piterkova et al., 2009; Sedlářvá et al., 2010; Melillo et al., 2011) during the early stages of infection. In incompatible interactions, ROS was detected in the stomata and the necrotic mesophyll cells following fungal penetration and the induction of HR (Wang et al., 2007, 2010). Similar results were obtained by our laboratory with regard to NO (Yin et al., unpublished). The generation of ROS and NO is also involved in systemic acquired resistance (Gao et al., 2014; Wendehenne et al., 2014).

Combining all of the results, we deduced that in addition to supplying itself for apical growth, it was likely that *Pst* also produced a small amount of ROS and NO to adapt to the highly oxidative conditions in the infected plant. The small amount of ROS and NO might protect the growth and development of the germ tube and the expansion of hyphae in the intercellular space from the harm of highly oxidative condition in the infected plant.

In future studies, a higher priority should be given to defining the molecular identity of the genes involved in NO and ROS biosynthesis and the relationship between NO and ROS in signaling transduction during germination and germ tube growth in *Pst*.

AUTHOR CONTRIBUTIONS

ZK and HZ designed experiments; SY carried out experiments; ZG analyzed experimental results; CW and LH joined the discussion and gave the original ideas; SY wrote the manuscript.

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frontiers in Plant Science

REVIEW
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When Bad Guys Become Good Ones: The Key Role of Reactive Oxygen Species and Nitric Oxide in the Plant Responses to Abiotic Stress

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The natural environment of plants is composed of a complex set of abiotic stresses and their ability to respond to these stresses is highly flexible and finely balanced through the interaction between signaling molecules. In this review, we highlight the integrated action between reactive oxygen species (ROS) and reactive nitrogen species (RNS), particularly nitric oxide (NO), involved in the acclimation to different abiotic stresses. Under stressful conditions, the biosynthesis transport and the metabolism of ROS and NO influence plant response mechanisms. The enzymes involved in ROS and NO synthesis and scavenging can be found in different cells compartments and their temporal and spatial locations are determinant for signaling mechanisms. Both ROS and NO are involved in long distances signaling (ROS wave and GSNO transport), promoting an acquired systemic acclimation to abiotic stresses. The mechanisms of abiotic stresses response triggered by ROS and NO involve some general steps, as the enhancement of antioxidant systems, but also stress-specific mechanisms, according to the stress type (drought, hypoxia, heavy metals, etc.), and demand the interaction with other signaling molecules, such as MAPK, plant hormones, and calcium. The transduction of ROS and NO bioactivity involves post-translational modifications of proteins, particularly *S*-glutathionylation for ROS, and *S*-nitrosylation for NO. These changes may alter the activity, stability, and interaction with other molecules or subcellular location of proteins, changing the entire cell dynamics and contributing to the maintenance of homeostasis. However, despite the recent advances about the roles of ROS and NO in signaling cascades, many challenges remain, and future studies focusing on the signaling of these molecules *in planta* are still necessary.

Keywords: crosstalk, signaling, systemic acquired acclimation, *S*-nitrosylation, *S*-glutathionylation, gene expression

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INTRODUCTION

A typical plant cell has more than 30,000 genes and a large number of proteins, many of which are still unknown, and these proteins may have their activity and/or function altered by several types of post-translational modifications (Cramer et al., 2011). Therefore, at the cellular level, plant responses to the environment are extremely complex and involve interactions and crosstalk with

many molecular pathways. One of the first plant responses to the environment involves reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are key signaling molecules and regulate many different plant processes through the activation of secondary messengers, the induction of gene transcription and changes in enzyme activity (Gaupels et al., 2011; Mengel et al., 2013; Lamotte et al., 2015).

Reactive oxygen species is a generic term used to describe chemical species formed from the incomplete reduction of molecular oxygen. The best-known ROS include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-; **Table 1**). ROS have distinct biological properties, a short half-life and high chemical reactivity (OH^- has indiscriminate reactivity to biological molecules, while $O_2^{\bullet -}$ and H_2O_2 have preferred biological targets; del Río, 2015). Similarly, RNS is a term used to collectively refer to nitric oxide (NO) and the molecules derived from this radical (**Table 1**) (Patel et al., 1999; Rahman et al., 2012). NO is a gaseous, small, reactive molecule that readily diffuses across the cells and interacts with different cellular compounds, including other radicals (Correa-Aragunde et al., 2015). Due to their high reactivity and potential to damage cellular structures under conditions of redox imbalance, the generation of ROS and RNS in cells was originally considered to be a uniquely harmful and damaging process (Demidchik, 2015; Lushchak, 2015). Currently, however, it is known that these molecules are important components of signaling networks in various plant processes, which is possible due to the development of effective antioxidant systems that are capable, in most cases, of containing the toxicity of ROS and RNS, allowing these molecules to act as efficient signal transducers (del Río et al., 2006; del Río, 2015).

Nitric oxide and ROS are involved in and interact with each other in a wide range of cellular processes, which include response to abiotic stresses (Joudoi et al., 2013), defense against pathogens (Asai et al., 2008) and normal growth and development processes, such as germination and flowering (El-Maarouf-Bouteau and Bailly, 2008). It is easy to see, therefore, that the changes triggered by these signaling molecules are highly variable according to the environmental context. Due to the high complexity of this process, there is still much that is unclear about the signaling mechanisms triggered by ROS and NO, the interaction of these molecules with each other and with other components of the signaling pathway, and the balance between production and elimination of reactive species by antioxidants. A growing number of studies have sought to answer these questions, and many advances have been made in the field. Thus, considering the central role of these molecules in the response and adaptation of plants to changes in the environment, the present review aims to summarize the existing knowledge of the interactions between ROS and NO in the plant response to abiotic stress, focusing on the sources and production sites of these molecules, interactions with other signaling components and molecular aspects.

TABLE 1 | Main reactive oxygen species (ROS) and reactive nitrogen species (RNS) found in plant cells (adapted from Rahman et al., 2012).

Free radicals	Non-radicals
Reactive oxygen species	
Superoxide, $O_2^{\bullet -}$	Hydrogen peroxide, H_2O_2
Alkoxyl, $RO^{\bullet}$	Hypochlorous acid, HOCl
Hydroxyl, OH^-	Ozone, O_3
Peroxyl, $ROO^{\bullet}$	Peroxynitrite, $ONOO^-$
Hydroperoxyl, $HO_2^{\bullet}$	Singlet oxygen, $^1O_2$
Reactive nitrogen species	
Nitric oxide, $NO^{\bullet}$	Nitrous acid, HNO_2
Nitric dioxide, $NO_2^{\bullet}$	Nitrosonium cation, NO^+
Nitrate radical, $NO_3^{\bullet}$	Nitrosyl anion, NO^-
	Peroxynitrite, $ONOO^-$
	Alkylperoxynitrites: ROONO
	Dinitrogen trioxide, N_2O_3

BIOSYNTHESIS, TRANSPORT, AND METABOLISM OF ROS AND NO

During cell signaling in response to stress, the redox state of the plant cells is rapidly altered by both the increase in ROS and NO and the inactivation of antioxidant enzymes (del Río, 2015). As a result, the concentration of these reactive species is suddenly elevated, which is necessary to trigger specific cellular responses. These responses include defense mechanisms to abiotic stresses, such as increased concentration and activity of antioxidant systems (Shi et al., 2014) or programmed cell death, which is important to eliminate cells that have been severely damaged (Yun et al., 2011). The enzymes involved in the synthesis of ROS and NO can be found in different cellular compartments (**Figure 1**), and their temporal and spatial localization is critical for signaling (Groß et al., 2013). Indeed, ROS and NO have unique roles based on their compartment of origin, which is probably due to interactions with local molecules in each organelle (Møller and Sweetlove, 2010; Shapiguzov et al., 2012; Mur et al., 2013). It has been observed, for example, that the transcriptional changes mediated by H_2O_2 produced in the apoplasts are distinct from the gene expression responses triggered by H_2O_2 produced in the chloroplasts (Gadjev et al., 2006; Sierla et al., 2013). Similarly, the NO generated from the plasma membrane is important in hypoxic conditions, whereas the NO generated from the chloroplasts and mitochondria is involved in the response to heavy metals (Kumar and Trivedi, 2016).

The plasma membrane is the main site of ROS production due to activity from proteins belonging to the NADPH oxidase family (respiratory burst oxidase homolog, RBOH). NADPH oxidases are integral membrane proteins that promote the transfer of cytoplasmic NADPH electrons to extracellular oxygen, forming $O_2^{\bullet -}$ and promoting ROS accumulation in the apoplast (Das and Roychoudhury, 2014). Several studies have shown that stressful conditions stimulate the expression and activity of NADPH oxidases, leading to an oxidative burst

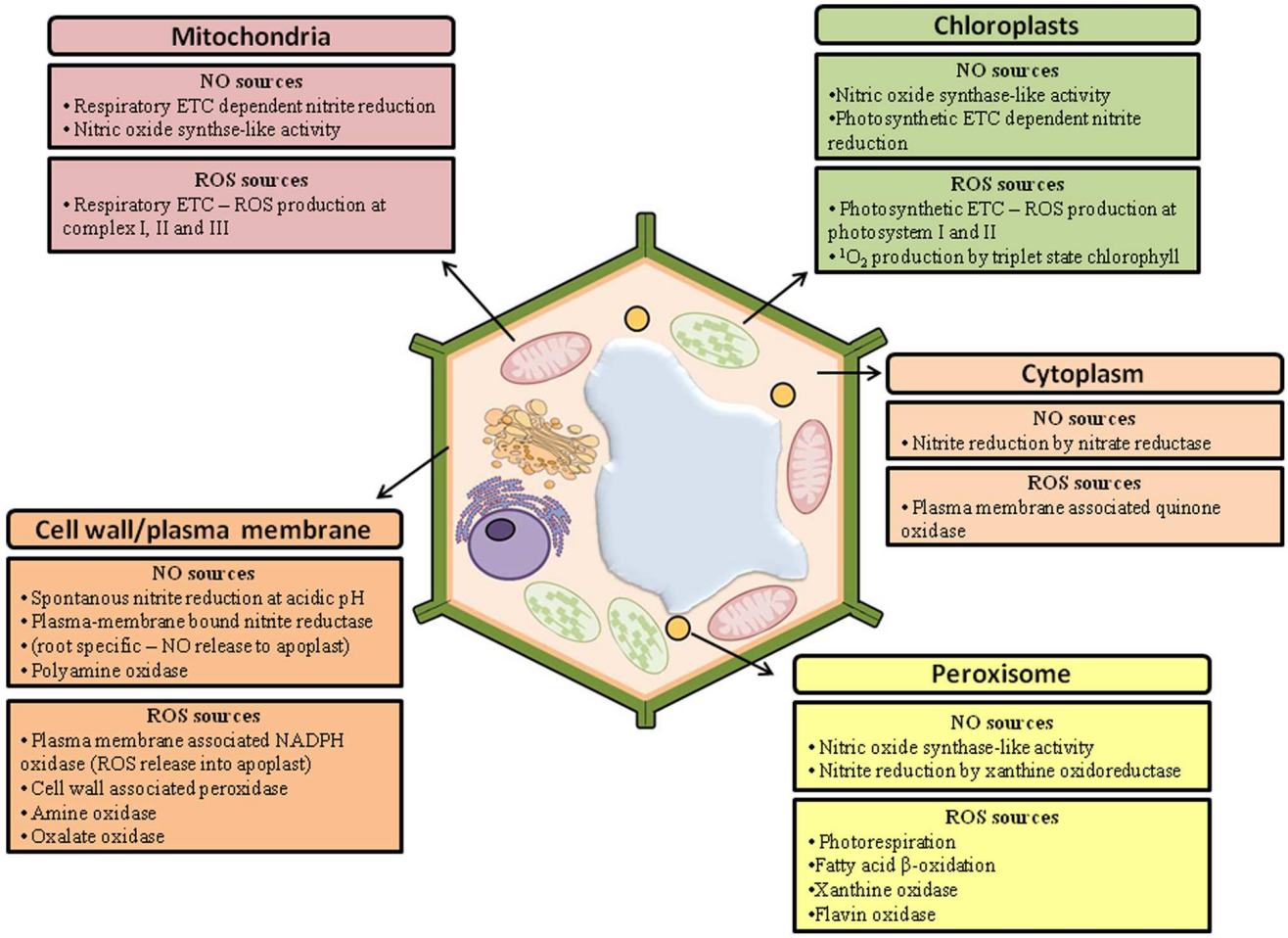

FIGURE 1 | Main sources of nitric oxide (NO) and reactive oxygen species (ROS) in plant cells (adapted from Groß et al., 2013).

(Jajic et al., 2015; Wang X. et al., 2015). Other oxidases and peroxidases associated with the cell wall are also involved in the generation of ROS in the apoplast, although their involvement in the response to stressors is not well defined (Das and Roychoudhury, 2014). In addition to promoting specific signaling events, which involve interactions with local signals, the RBOH-mediated oxidative burst of ROS production triggers the production of ROS in neighboring cells, initiating a long distance signaling event called a ROS wave. Each cell along the ROS wave activates their own RBOH proteins, generating a systemic wave of propagation of ROS production, which travels through the apoplast from the initial tissue to whole plants at rates of up to 8.4 cm min^{-1}, promoting systemic acquired acclimation (SAA; Mittler and Blumwald, 2015). SAA enables all plant cells, not just those who first perceived the external stimulus, to alter their gene expression and metabolism in response to the stressor. Although the ROS wave is necessary for SAA, the response elicited is not always specific to the stress that initiated the signaling process, suggesting that the main function of the ROS wave is to prepare the plant for SAA and that other signals are required to mediate stress-specific SAA (Gilroy et al., 2014).

In addition to the apoplast, various cellular organelles, such as chloroplasts and mitochondria, also generate ROS. In fact, when illuminated, chloroplasts are important sources of ROS due to the intense electron transport during photosynthesis and the release of oxygen in PSII (Gupta and Igamberdiev, 2015). In mitochondria, ROS production occurs when the transfer of electrons exceeds the capacity of the alternative oxidase and the cytochrome oxidase to eliminate excess electrons, resulting in their transfer to molecular oxygen, mainly from complexes I and III. Another organelle involved in ROS synthesis in stressful conditions is the peroxisome. Peroxisomes generate $O_2^{\bullet -}$ and H_2O_2 as a result of their metabolic activity, which involves processes such as photorespiration, the glyoxylate cycle, and β-oxidation (Tripathy and Oelmüller, 2012). These different pools of ROS, produced in distinct compartments, communicate with each other in the cells to regulate the plant metabolism. It is believed, for example, that the signal generated by the oxidative burst in the apoplast is transduced to chloroplasts, where a second wave of ROS generation is initiated (Shapiguzov et al., 2012).

This signal transduction probably involves cytosolic components as well as the transport of ROS through the lipid bilayer (the O_2^- produced in the apoplast can be converted to H_2O_2, which enters the cell through the aquaporins) or signal detection by apoplastic proteins and membrane receptors (de Dios Barajas-López et al., 2013). ROS generated in the chloroplasts, in turn, are involved in the retrograde signal from the chloroplast to the nucleus and influence the expression of many defense genes, in addition to inhibiting the transcription of genes associated with photosynthesis (de Dios Barajas-López et al., 2013). This has also been observed in other organelles, such as peroxisomes, where ROS accumulation can alter gene transcription (Sandalio and Romero-Puertas, 2015).

The maintenance of ROS levels also involves the participation of antioxidant mechanisms, which are associated with the elimination of these reactive species and can be divided into enzymatic and non-enzymatic mechanisms. Among enzymatic antioxidants, superoxide dismutase (SOD) is especially important because it catalyzes the removal of $O_2^{\bullet-}$, the first ROS formed after exposure to various stressors. Other antioxidant enzymes include ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT), which convert H_2O_2 to water (Lázaro et al., 2013). In combination with these enzymes, non-enzymatic antioxidants, such as glutathione, ascorbate, and tocopherol, also play a crucial role in maintaining ROS levels by acting as redox buffers in plant cells. Although the synthesis of these antioxidant molecules may, at first, be inhibited to allow the occurrence of the oxidative burst (del Río, 2015), once the signal is initiated, these mechanisms are activated and function cooperatively (Viehweger, 2014).

In contrast to ROS, the mechanisms of NO synthesis in plant cells are not yet fully understood, constituting one of the major challenges to studies investigating this signaling molecule. However, several biosynthetic pathways for NO have been proposed (**Figure 1**), which can be divided into reductive pathways, including the action of xanthine oxidoreductase in the peroxisomes (Millar et al., 1998) and nitrite:NO reductase attached to the membrane (Stöhr et al., 2001); and oxidative pathways, such as the pathways mediated by hydroxylamines (Rümer et al., 2009) and polyamines (Filippou et al., 2013). Apparently, the action of nitrate reductase (NR), a cytosolic enzyme essential for the assimilation of nitrogen, also represents an important source of NO for plants (Horchani et al., 2011). It has been suggested that NR is involved in the production of NO during a variety of physiological processes, such as bacterial defense (Modolo et al., 2005; Mur et al., 2013), hypoxia (Igamberdiev and Hill, 2004), cold (Zhao et al., 2009), drought (Freschi et al., 2010), and various aspects of development, such as floral transition and the formation of lateral roots (Seligman et al., 2008; Mur et al., 2013). However, under normal growth conditions, NR preferentially reduces nitrate to nitrite, and NR is only able to generate significant amounts of NO under certain conditions, such as anaerobic conditions or high concentrations of nitrite (Gupta et al., 2011; Mur et al., 2013). There have been numerous reports of an arginine-dependent nitric oxide synthase (NOS) in extracts of different plant species (Jasid et al., 2006; Zhao et al., 2007; Gas et al., 2009; del Río, 2011), but its presence in plants has not been unequivocally demonstrated (Domingos et al., 2015; Gupta and Igamberdiev, 2015).

In addition to biosynthetic processes, another crucial factor in NO concentration in the cell is the formation of *S*-nitrosothiols, particularly *S*-nitrosoglutathione (GSNO), relatively stable molecules in solution that may act as reservoirs of NO (Leterrier et al., 2011). GSNO is formed by *S*-nitrosylation of glutathione (GSH) by NO and can be transported in the phloem, thus contributing to the transport of this signaling molecule over long distances, which plays an important role in SAA (Arasimowicz-Jelonek et al., 2014). GSNO also regulates the NO concentration in the cell via inhibition of the nitrogen assimilation pathways (Fungillo et al., 2014). GSNO turnover is controlled by GSNO reductase (GSNOR), which catalyzes the deamination of GSNO into glutathione disulfide (GSSG) and NH_3. Thus, GSNOR regulates the cellular levels of GSNO and is important in maintaining homeostasis of NO, which is essential for transient cell signaling (Malik et al., 2011). The levels and activity of GSNOR are modulated in conditions of stress and are determined, among other factors, by the balance between ROS and NO (Cheng et al., 2015; Wang D. et al., 2015; Yang et al., 2015).

Degradation of NO is as important as synthesis and transport in determining the final concentration of this signal molecule in plant cells. Recently, Sanz-Luque et al. (2015) demonstrated that the green alga *Chlamydomonas reinhardtii* has a specific mechanism for the elimination of NO, which involves truncated hemoglobin THB1. The authors verified that THB1 has NO dioxygenase activity (produces NO_3^- from NO and O_2) and maintains in its active form through a mechanism that removes electrons from NR and alters its activity. Another class of hemoglobins, the non-symbiotic hemoglobins (nsHb), particularly those belonging to the GLB1 class, have also been reported to have NO dioxygenase activity and to promote the degradation of NO in certain circumstances, such as hypoxia (Perazzolli et al., 2004). The reduction in *GLB1* expression, moreover, allows NO concentration to increase, triggering defense responses against stress (Mur et al., 2012). The interaction between NO and $O_2^{\bullet-}$, which generates peroxynitrite ($ONOO^-$), is also regarded as a mechanism of NO elimination and involves the modulation of mitochondrial activity (Wullf et al., 2009).

The biosynthesis and degradation of ROS and NO influence each other. ROS are well-known inducers of NO synthesis in various plant species exposed to abiotic stress, although the signaling involved in this process is still not completely understood. NO, in turn, limits the accumulation of ROS by inhibition of NADPH oxidases, as well as by promoting changes in the antioxidant systems, suggesting the existence of complex feedback regulation of both signaling molecules (Groß et al., 2013). In fact, the activation of antioxidant mechanisms to maintain ROS homeostasis often involves the participation of NO (Farnese et al., 2013; Shi et al., 2014; Silveira et al., 2015). It has been shown that the addition of NO increased the activity of SOD up to 110% in sorghum plants exposed to arsenic (Saxena and Shekhawat, 2013), in addition to the increase in CAT and APX and the activation of the ascorbate-glutathione

cycle (Hasanuzzaman and Fujita, 2013; Shi et al., 2014; Cheng et al., 2015). Some studies, however, have suggested that NO can inhibit the antioxidant capacity of the cell (Marti et al., 2013). These seemingly contradictory results may be due to the dose-dependent effects of NO on cellular redox status. According to this hypothesis, low concentrations of NO stimulate the antioxidant system and promote adaptation to stress conditions, while high concentrations of NO trigger severe cell damage and even cell death (Thomas et al., 2008; Groß et al., 2013).

MOLECULAR BASES OF ROS AND NO ACTION

The mode of action of ROS and NO at the molecular level was and still is the subject of many studies, both in plants and other organisms, such as mammals and bacteria (Green et al., 2014; Lamotte et al., 2015; Morales et al., 2015). The available data indicate that the effects of NO, as well as certain species derived from this molecule, depend on chemical changes in proteins, which can occur by three different mechanisms: metal nitrosylation, tyrosine nitration, and *S*-nitrosylation (Lamotte et al., 2015). Metal nitrosylation consists of NO binding to transition metals in metalloproteins. Soluble guanylate cyclase is an example of an enzyme that is modulated by this type of post-translational modification. Tyrosine nitration is the addition of a nitro group to tyrosine residues. Tyrosine nitration is carried out mainly by peroxynitrite ($ONOO^-$), the product of the reaction between NO and $O_2^{\bullet-}$. Although tyrosine nitration was originally considered indicative of stress, recent evidence suggests its involvement in cell signaling (Mengel et al., 2013). Finally, *S*-nitrosylation, which consists of NO binding to cysteine residues in target proteins, is apparently the principal mechanism for the transduction of the NO bioactivity. *S*-nitrosylation can also occur via *trans*-nitrosylation, that is, by the transfer of NO from an *S*-nitrosylated residue to another thiol group through the action of low-molecular weight nitrosothiols, such as GSNO (Lamotte et al., 2015). Regardless of the mechanism involved, *S*-nitrosylation is a post-translational modification that can alter the activity, stability, conformation, interactions with other molecules or subcellular localization of the target protein, regulating a wide range of cellular functions and signaling events (Sevilla et al., 2015).

S-nitrosylation is an important process in plant responses to abiotic stress. Exposure to salt stress, for example, results in the *S*-nitrosylation of enzymes involved in different physiological processes, such as respiration, photorespiration, and antioxidant pathways (Camejo et al., 2013), while in plants exposed to low temperatures, the enzymes involved in carbon metabolism were the main group of *S*-nitrosylated proteins (Puyaubert et al., 2014). *S*-nitrosylation of proteins that participate in central processes in the plant cell presumably contributes to the metabolic reprogramming required to maintain homeostasis under stress conditions. In addition to changes in cellular enzyme dynamics, *S*-nitrosylation may also trigger changes in gene expression as a result of *S*-nitrosylation of transcription factors, affecting their affinity for DNA or their location. Recently, it was demonstrated that *S*-nitrosylation is a negative regulator of transcription factors from the MYB family (regulator of tolerance to biotic and abiotic stresses), which may be important for the inactivation of this regulatory protein after the initial response of plants to stress (Tavares et al., 2014). Several *S*-nitrosylated nuclear proteins have also been identified, including histone deacetylases, which highlights the regulatory role of NO in events located in the nucleus (Chaki et al., 2015). Histone deacetylases are responsible for the removal of acetyl groups on histones, promoting the chromatin condensation, which makes the genes less accessible to the transcriptional machinery (Mengel et al., 2013). In mammalian cells, *S*-nitrosylated histone deacetylases become detached from the chromatin, increasing acetylation and gene expression (Nott et al., 2008). Thus, the *S*-nitrosylation of deacetylases suggests that NO participates in the regulation of epigenetic processes in plants (Floryszak-Wieczorek et al., 2012; Chaki et al., 2015).

The *S*-nitrosylation state of any protein is determined by the balance between nitrosylation and denitrosylation reactions. In fact, denitrosylation, which involves the removal of NO from cysteine residues, is essential for the reversibility of *S*-nitrosylation and influences the enzyme activity, protein–protein interactions and many other aspects of signaling (Sevilla et al., 2015). Although this process has been more extensively studied in mammals, recent evidence has shown that it occurs in plant cells (Kneeshaw et al., 2014; Benhar, 2015). Among the molecules which may be involved in the denitrosylation process, the GSNO/GSNOR (discussed earlier in this review) and the thioredoxin/thioredoxin reductase (Trx/TR) systems are essential for the maintenance of homeostasis of nitrosothiols in plants (Lamotte et al., 2015). In the Trx/TR system, Trx reduces nitrosothiols through its dithiol moiety, generating free thiol groups and oxidized Trx. Regeneration of Trx occurs through the action of TR and NADPH (Benhar, 2015). Thus, the control of the redox status of thiol groups depends on their interaction with NO and with the denitrosylation systems, which influences the intensity and duration of the signaling events (Benhar, 2015).

As observed for NO, ROS also transmit signals via post-translational modifications in proteins and, once more, cysteine residues are the main targets. However, while NO promotes *S*-nitrosylation, ROS can trigger a diverse range of oxidative post-translational modifications (Ox-PTM), reversible or irreversible, including *S*-glutathionylation, disulfide bond formation, and sulfhydration (Akter et al., 2015). A single protein can undergo different types of Ox-PTMs, and there is evidence that each Ox-PTM may have a distinct biological role (Couturier et al., 2013). The first step in ROS-dependent signaling involves the reverse oxidation of a cysteine residue, forming sulfenic acid (R-SOH). This modification is highly unstable and will lead to subsequent changes; the major ones are the reaction with free protein thiols to form disulfide bonds or the covalent attachment of low-molecular weight thiols, such as GSH, promoting *S*-glutathionylation, a process that is important in signaling and protein protection against superoxide. The reduction of disulfide bonds and deglutathionylation interrupt the signal that initiated with the Ox-PTMs and are controlled by glutaredoxins and thioredoxin, respectively (Waszczak et al., 2014, 2015).

The Ox-PTMs, particularly *S*-glutathionylation, play a central role in the response to abiotic stresses and can modulate numerous cellular processes affecting proteins, transcription factors, and chromatin structure. These mechanisms, however, have mostly been studied in animals and bacteria, and many aspects of the Ox-PTM-mediated responses are unknown in plants (Zagorchev et al., 2013). One example of Ox-PTMs mediating changes in cellular dynamics is the transcription factor ERFVII (ethylene-responsive group factor VII), which is important in altering gene expression under hypoxic conditions. ERFVII is bound to the plasma membrane and is only released in low-oxygen conditions. Its translocation to the nucleus activates the expression of hypoxia-responsive genes. In the presence of oxygen, however, ERFVII cysteine residues are oxidized to sulfenic acid, conjugated with arginine and directed to degradation, down-regulating the expression of genes that are no longer needed (Dietz, 2014). The Ox-PTMs can also be positive regulators of gene transcription, as in the case of transcription factors of heat shock proteins (HSF), whose oxidation by H_2O_2 induces translocation from the cytosol to the nucleus (Habibi, 2014). Despite the growing number of studies, however, there is still little information about the effects of *S*-nitrosylation and Ox-PTMs on gene expression and the consequences of these changes on plant metabolism in stress conditions. Thus, the molecular mechanisms involved in cell signaling mediated by ROS and NO are still far from being fully understood.

CROSSTALK BETWEEN ROS AND NO IN THE RESPONSE TO ABIOTIC STRESS

Heavy Metals

Traditionally, heavy metals are considered those chemicals that have a density higher than 5 g cm^{-3} or an atomic number higher than 20. In plant physiology, however, the term heavy metal has been used generically to refer to any metal or metalloid that is toxic to plants, even when present at low concentrations (Singh et al., 2011; Oz et al., 2015). Although some of the members of this group are necessary for growth and development, others have no known function in plant cells and, regardless of their physiological role, the accumulation of metals usually results in severe cell damage, which can lead to the death of the plant (Besson-Bard et al., 2009). Heavy metals enter plant cells by transporters present in the plasma membrane and may be retained in the roots or transported to the shoots, according to the cellular detoxification mechanisms in each species (Tangahu et al., 2011). In general, the defense mechanisms of plants to heavy metals can be divided into two groups, which may occur simultaneously: (i) regulation of the concentration of free metal in the cytosol through metal exclusion, metal binding to the cell wall or compartmentalization in the vacuole; and (ii) physiological, biochemical, and molecular changes that allow the reprogramming of plant metabolism and the maintenance of cellular homeostasis.

Recent studies have indicated that the interaction between NO and ROS is essential for tolerance to heavy metals (**Figure 2**) (Wang et al., 2014; Feigl et al., 2015; Silveira et al., 2015; Thao et al., 2015). The exact signaling mechanisms involved in this process, however, have not been clarified, and many questions remain unanswered. Currently, it is known that increases in ROS are one of the first cellular signals in response to excess heavy metals. In fact, heavy metals can activate the production of ROS in the apoplast, stimulating NADPH oxidases, and in organelles such as chloroplasts and mitochondria (Chmielowska-Bak et al., 2014). Heavy metals enhance the synthesis of NO by mechanisms that vary according to the chemical characteristics of the metal. Lead, for example, apparently increases the activity of cytosolic NR (Yu et al., 2012), while increases in NO levels mediated by cadmium are related to iron-induced deficiency (Besson-Bard et al., 2009). Interestingly, some metalloids, such as arsenic, stimulate both the synthesis of NO and the activity of GSNOR, and the balance between these two processes will determine the final concentration of the signaling molecule (Leterrier et al., 2012). In addition, the increase in NO may result from signaling triggered by excessive ROS following exposure to metals (Yun et al., 2011).

According to their concentrations, ROS and NO can cause oxidative/nitrosative stress in cells or may act as signaling molecules. At low concentrations, NO contributes to increased tolerance of the plants to metals in various ways, for example, by promoting metal binding to the cell wall, preventing their entry into the cell (Singh et al., 2011) or promoting their compartmentalization in the vacuole, either by increasing phytochelatin synthesis (De Michele et al., 2009) or by altering the activity of proton pumps in the vacuolar membrane to create an electrochemical gradient that favors the absorption of metals (Cui et al., 2010). In addition to these effects, NO can also reprogram plant physiological processes and stimulate the synthesis and activity of antioxidant systems, which is essential to limit the oxidative stress induced by metals (Cheng et al., 2015; Andrade et al., 2016). Finally, post-translational changes triggered by NO can decrease the activity of enzymes involved in ROS metabolism, such as glycolate oxidase and NADPH oxidase, allowing the cell to re-establish redox homeostasis (Yun et al., 2011; Quiang et al., 2012).

Drought

Water deficits are the main environmental factor limiting the growth and productivity of plants worldwide. Indeed, the damage triggered by drought may be greater than the damage caused by other biotic and abiotic factors combined (Chaves et al., 2009). Plants tolerant to drought usually have a strict control of stomatal movements and a fine balance of cellular metabolism, and both ROS and NO are important in these processes (**Figure 2**) (Osakabe et al., 2014). Drought stress-induced NO is found in a wide variety of plant species, including vegetables, horticultural plants and epiphytes, suggesting the universal requirement of NO during drought stress signaling (Santisree et al., 2015). The metabolic pathways involved in this process, however, are still unclear, although evidence suggests the involvement of NR (Arasimowicz-Jelonek et al., 2009) and xanthine oxidoreductase (Yu et al., 2014). The generation of ROS during drought, on the other hand, is well known and commonly involves changes in plant metabolic processes. For

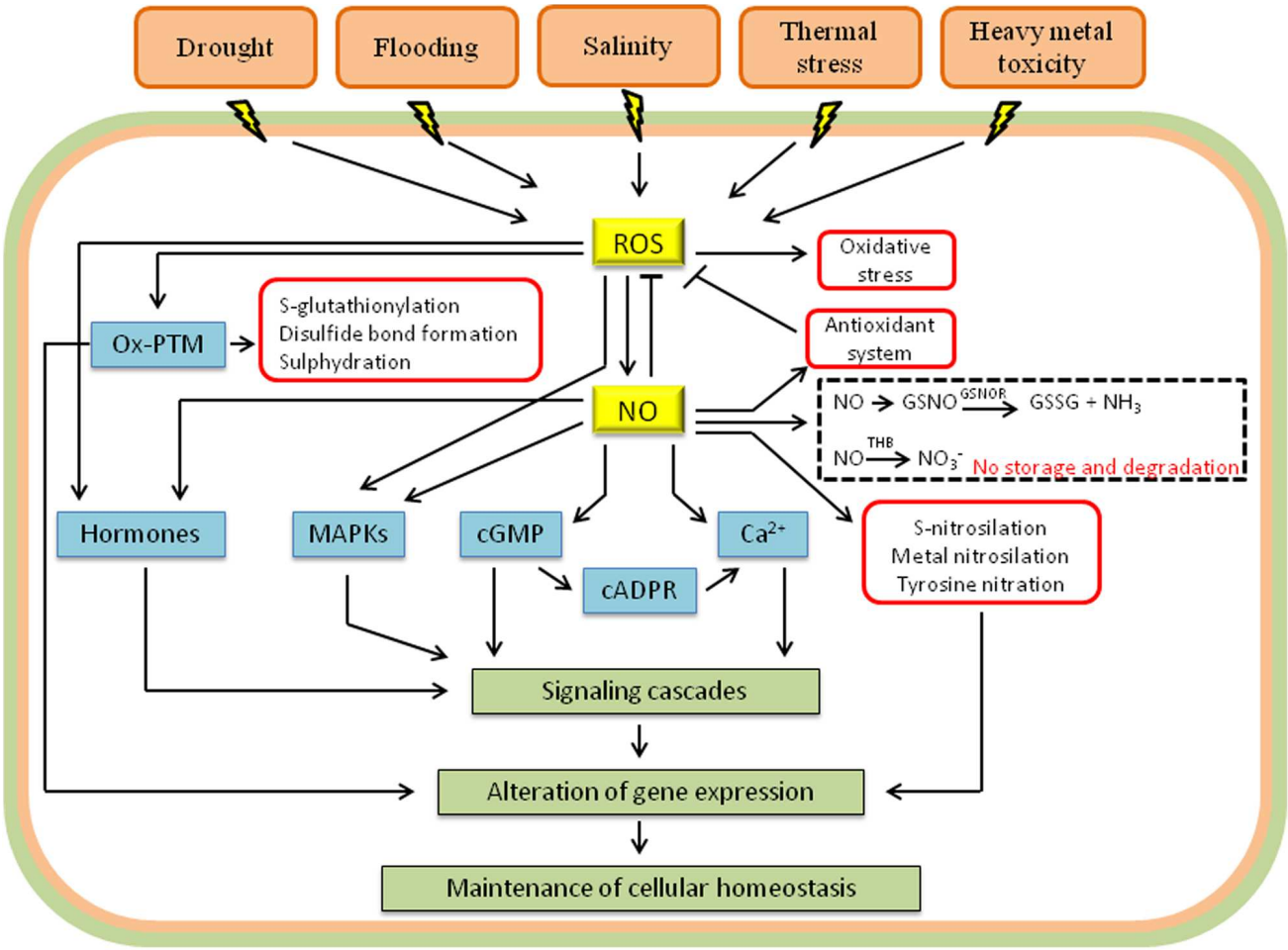

FIGURE 2 | Schematic representation showing the interplay between NO, ROS, and other signaling molecules in response to abiotic stress. The signaling molecules include hormones, mitogen-activated protein kinases (MAPKs), cyclic guanosine monophosphate (cGMP), cyclic adenosine diphosphoribose (cADPR) and calcium (Ca^{2+}). Besides the interaction with components of signaling pathways, NO and ROS also transmit signals via post-translational modifications in proteins [metal nitrosylation, tyrosine nitration, *S*-nitrosylation, and oxidative post-translational modifications (Ox-PTM)]. ROS degradation by antioxidants and NO storage (GSNO, *S*-nitrosoglutathione) and degradation by GSNOR (GSNO reductase) and THB (truncated hemoglobin) are demonstrated. *Arrows* and *T-bars* indicate activation and inhibition, respectively (adapted from Oz et al., 2015).

example, drought may reduce the activity of Rubisco (Parry et al., 2002), which compromises the fixation of CO_2 and the regeneration of $NADP^+$ via the Calvin cycle. This results in an over-reduction of the electron transport chain in the chloroplasts and, consequently, the leakage of electrons to O_2, mainly by the Mehler reaction and photorespiration, resulting in the generation of ROS (Carvalho, 2008). In parallel with the physiological changes, NADPH oxidases are also involved in the generation of ROS in water stress conditions, as they are essential for the activation of defense responses against drought (Wang X. et al., 2015).

One of the first and most important physiological responses induced by drought is the reduction of the stomata opening (Neill et al., 2008). In water stress conditions, the complex dynamics of stomatal movement are directly related to the concentration of abscisic acid (ABA), ROS, and NO. In this process, ABA, an important plant hormone traditionally associated with responses to water stress, acts as an upstream regulator, inducing the synthesis of NO. NO and ROS, in turn, act synergistically to mediate stomatal closure through the formation of 8-nitro-cGMP (Joudoi et al., 2013). ABA is also involved in several other plant responses to water stress, for example, the induction of gene expression and the synthesis of defense compounds, such as proline. Increasing only ABA, however, is not sufficient to induce the synthesis of these compounds, as observed after exogenous application of ABA to *Arabidopsis thaliana* (Verslues and Bray, 2006), suggesting that factors other than ABA are required to modulate the response to drought, possibly the cellular redox status. Thus, the production of ROS and the subsequent change in the redox state of the cell have been suggested as factors required to initiate signal transduction mediated by ABA (Carvalho, 2008).

Similar to ABA and ROS, NO has been shown to alter gene expression in response to water deficits. Transgenic *Arabidopsis* lines that constitutively express rat neuronal NO synthase

showed changes in gene expression relative to wild plants when subjected to drought, with 184 genes up-regulated and 263 down-regulated. The main transcriptional changes were observed in the genes involved in redox metabolism and sugar metabolism and in transcription factors. These transcriptional changes were accompanied by higher survival rates and high biomass production, indicating a protective effect of NO (Shi et al., 2014). Moreover, it has been reported that NO may trigger epigenetic modifications, such as DNA methylation, in response to water stress conditions. Indeed, it was observed that the exogenous application of SNP (an NO donor) decreases the overall methylation levels in *Dendrobium huoshanense*, leading to increases in the activity of antioxidant enzymes (Fan et al., 2012).

Flooding

Climate projections for the next decades, besides pointing out an increase in drought intensity and frequency for some regions, also highlight a significant increase in rainfall for others, especially those regions with tropical climate (Hirabayashi et al., 2013). If these predictions are confirmed, productivity for several crop areas and ecosystems can be significantly impacted due to flooding events (Bailey-Serres and Voesenek, 2008). Despite the fact that flooding has been studied to a lesser extent than drought, in recent years, researches about molecular mechanisms that promote flooding tolerance have progressed rapidly (Voesenek and Bailey-Serres, 2015). In general, the mechanisms that confer tolerance to such stress can be grouped into two categories: "escape," which involves anatomical and morphological modifications that allow access of the submerged cells to O_2 and CO_2, and "quiescence," which promotes a profound change in metabolism and growth of flooded plants (Voesenek and Bailey-Serres, 2015).

Several evidences have suggested that plants can sense the reduction of O_2 availability caused by flooding through different ways. Some of these mechanisms of perception may involve changes in the dynamics of ROS and NO production and consumption, because of mitochondrial electron transport inhibition (Rhoads and Subbaiah, 2007). In fact, studies in *Arabidopsis thaliana* showed that oxygen deprivation induces ROS production in the complex III, which triggers a transient activation of MAPK signaling cascade (Chang et al., 2012). In addition, regulatory responses induced by ROS involve, at least in part, the interaction with ethylene, an important plant hormone involved in plant response to various stresses. Indeed, in rice plants, ROS and ethylene were involved in the production of adventitious root in stem nodes (Steffens et al., 2012).

The origins of NO in hypoxic conditions are not clearly understood, although nitrite and ascorbate are apparently involved in this process, as well as mitochondrial reactions (Wang and Hargrove, 2013). NO generation in mitochondria is an important process at low oxygen conditions. In this process, the nitrite acts as an electron acceptor in complex IV, complex III and in the alternative oxidase, keeping a limited production of ATP when oxygen is not available and, thus, preventing the collapse of cellular energy status (Gupta et al., 2011). The produced NO diffuses into the cytosol, where it is converted to nitrate by hemoglobin action. Class 1 hemoglobins are proteins capable of binding to NO, and at low O_2 concentrations catalyze its conversion to nitrate, which can be converted to nitrite by NR (Dordas, 2015). Nitrite, in turn, can be converted again to NO in the mitochondria, closing the cycle (Gupta et al., 2011).

Salt Stress

Salinity is an ever-present threat to crop yields, especially in places where irrigation is required. In fact, studies show that approximately 20% of all cultivated areas in the world are affected by this type of stress, and this percentage is likely to increase due to inadequate irrigation practices (Muns and Tester, 2008). Salt stress compromises the intracellular ion homeostasis, which leads to membrane dysfunction, alteration of metabolic activities, growth inhibition, and even cell death (Zhang et al., 2006). Plants tolerant to salt stress have many diverse strategies to tolerate high concentrations of solutes. The principal mechanisms include, but are not limited to, (i) ion homeostasis and compartmentalization, (ii) ion transport and uptake, (iii) biosynthesis of osmoprotectants and compatible solutes, (iv) activation of antioxidant enzymes and synthesis of antioxidant compounds, and (v) synthesis of polyamines (Gupta and Huang, 2014). All these events are triggered and integrated into the plant cell through the action of signaling molecules, especially ROS, NO, and plant hormones (**Figure 2**) (Filippou et al., 2014).

Nitric oxide is essential for the tolerance of plants to high salt concentrations. Examples of the role of NO in this process can be observed in mutant plants of *Arabidopsis* (*Atnoa1*) which show deficiency in NO synthesis and hypersensitivity to salt stress (Zhao et al., 2007). Additionally, studies using NO donors and inhibitors showed that the ability of NO to alleviate stress was related to the change in the Na^+/K^+ ratio in the cytosol due to the increase in H^+-ATPase and H^+-PPase activity in the plasma and vacuolar membranes (Zhao et al., 2004; Zhang et al., 2006; Wang et al., 2009). In addition, NO is also able to induce the expression of defense genes against stress and significantly increase the activity of enzymes of the antioxidant system (Uchida et al., 2002). H_2O_2 also alters the expression of stress response genes in plants subjected to high salt concentrations, particularly in root cells, in addition to increasing the activity of specific enzymes (Miller et al., 2010). It is also important to note that the changes triggered by ROS and NO are not restricted to the tissues where these molecules were produced because addition of NO and H_2O_2 to the roots reduced the physiological imbalances caused by NaCl in leaves of *Citrus* plants (Tanou et al., 2009). This process is possible due to mechanisms such as the ROS wave and the transport of NO because both NO and GSNO have been observed in vascular tissues of plants exposed to salinity stress (Valderrama et al., 2007).

Another effect of NO and H_2O_2 in plants is the acquisition of immunity against salinity. This process, known as priming, describes the phenomenon in which plants previously subjected to a particular stress factor accelerate and potentiate their defense responses when subjected to that same stressor (Zhu, 2003; Zhang et al., 2004; Molassiotis et al., 2010). In fact, plants treated with low concentrations of NO and H_2O_2 had their metabolic and physiological responses potentiated when exposed to salt

stress. Likewise, NO-associated salt priming could be observed in halophytes and glycophytes that have increased tolerance to salinity when previously exposed to NO donors (Li et al., 2005). These data show that NO and H_2O_2 are priming agents that promote increased tolerance in the whole plant, minimizing the deleterious effects of subsequent exposure to salinity.

As noted in this review, different abiotic stresses have the same convergence point: they induce the production of ROS and NO. Thus, although each type of stress has its own specific characteristics, nearly all abiotic stressors alter the cellular redox state, and therefore, the participation of antioxidants in the plant response is essential. The activity and gene expression of antioxidants are strongly influenced, directly or indirectly, by changes in the concentrations of ROS and NO (Shao et al., 2008; Shi et al., 2014). In addition to the well-established role of the antioxidants in removing ROS, which has been discussed here, these molecules are important in signaling, providing essential information about the cellular redox status and influencing the expression of defense genes against biotic and abiotic stresses (Pastori et al., 2003; Foyer, 2005). Moreover, antioxidants are also needed to interrupt the signal transduction cascades when they are no longer needed, as is the case of glutaredoxin and thioredoxin (Waszczak et al., 2015).

Temperature Stress

Temperature, in combination with water deficiency, is one of the main abiotic factors that determine the survival and distribution of species worldwide. There are several reports in the literature indicating the adverse effects of high and low temperatures on the molecular, biochemical, and physiological characteristics of plants (Suzuki and Mittler, 2006). Although exposure to high and low temperatures triggers very distinct metabolic disorders, there is a common response between these two stressors: the increase in ROS, which damages cellular structures (**Figure 2**) (Potters et al., 2007; Zhou et al., 2012; Bita and Gerats, 2013). Similar to other abiotic stresses, increased levels of ROS following extreme temperatures appear to involve an imbalance between capturing and processing energy (Hasanuzzaman et al., 2013). However, over the past few years, several studies have shown that ROS and RNS, especially the interaction between them, are essential for acclimatization to high and low temperatures (Yu et al., 2014; Hossain et al., 2015). In fact, plants treated with low doses of NO and H_2O_2 perform better under conditions of thermal stress (Neill et al., 2002; Uchida et al., 2002; Abat and Deswal, 2009; Cantrel et al., 2011).

Exposure to high temperatures usually results in increased production of NO, a response that is important for acclimation to this type of stress (Leshem, 2000; Neill et al., 2003; Bouchard and Yamasaki, 2008; Yu et al., 2014). Indeed, addition of exogenous NO to plants subjected to high temperatures promotes the activation of enzymatic and non-enzymatic defense systems against ROS, reducing cellular damage (Song et al., 2006; Zhao et al., 2009; Hasanuzzaman et al., 2013). In addition, treatment with NO scavengers, such as cPTIO, reverses the beneficial effects caused by NO, which further reinforces the importance of this molecule in the tolerance to high temperatures (Song et al., 2006). Similarly, accumulation of ROS, especially H_2O_2, has been shown to be involved in signal transduction that culminates in increased expression of heat shock genes, which encode proteins that play critical roles in the maintenance of cellular homeostasis in stressful conditions (Königshofer et al., 2008).

As observed in high temperatures, exposure to cold can also promote a rapid increase in the endogenous levels of NO and ROS (Zhao et al., 2009; Cantrel et al., 2011). The increased production of NO in these conditions most likely involves an increase in NR because the *nia1nia2* mutants (NR-defective double mutants) showed lower concentrations of NO and increased susceptibility to cold stress (Cantrel et al., 2011). Tolerance to low temperatures mediated by NO involves the reprogramming of gene expression because it has been shown that NO production, induced by NR, promotes the transcription of the *P5CS1* and *ProDH* genes, along with accumulation of proline and an increased tolerance to cold (Zhao et al., 2009). In addition to the effects mediated by NO, the role of H_2O_2 in cold acclimation has also been widely documented (Prasad et al., 1994; Yu et al., 2003; Hung et al., 2007; Wang et al., 2010). Several studies indicate that exogenous application of H_2O_2 reduces cellular damage caused by low temperatures, increasing the survival rates (Neill et al., 2003). Again, this beneficial effect of H_2O_2 appears to involve an increase in the effectiveness of both the enzymatic (increased activity of the enzymes APX, GPX, and CAT) and non-enzymatic antioxidant mechanisms (increases in GSH levels; İseri et al., 2013; Wang et al., 2013). The signaling cascade responsible for the attenuation of cold stress involves, at least partially, transient increases in the cytosolic Ca^{2+} concentration due to H_2O_2-mediated activation of Ca^{2+} channels in the plasma membrane (Knight et al., 1996). However, although these results clearly indicate that ROS and NO are key molecules in the tolerance to high and low temperatures, few studies have focused on the molecular aspects and the signaling cascades responsible for these processes.

INTERACTIONS BETWEEN ROS, NO, AND OTHER SIGNALING MOLECULES IN THE RESPONSE TO ABIOTIC STRESS

The signaling pathways that respond to environmental stresses constitute intricate molecular networks that involve many other components besides ROS and NO, such as calcium (Ca^{2+}), cyclic nucleotides, plant hormones, and mitogen-activated protein kinases (MAPKs; **Figure 2**). The MAPK cascades, for example, are activated by ROS, NO, and hormones, representing a convergence point for these signaling molecules. MAPK signal transduction involves a phosphorylation cascade comprising MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and finally, MAPK, which is activated after phosphorylations of threonine and tyrosine residues in the conserved motif T-X-Y (Rodriguez et al., 2010). The interaction between ROS, NO, and MAPKs has been demonstrated in the plant response to various stressors, such as heavy metals (Chmielowska-Bak et al., 2014), drought (Wang et al., 2014), and osmotic stress (Xu et al., 2011). In *Arabidopsis*, high levels of ROS, especially H_2O_2, induce the transcription of the genes *OXI1* (Rentel et al., 2004) and *ANP1*

(Kotvun et al., 2000), which encode protein kinases required for full activation of the MAPKs. NO acts simultaneously with ROS in the activation of MAPKs, although the mechanisms involved in this process are not completely understood. In mammals, the activation of MAPK cascades by NO occurs indirectly and involves an increase in the synthesis of cGMP (Francis et al., 2010). It is possible that a similar mechanism is present in plants because it has been demonstrated that NO triggers the increases in the concentration of cyclic nucleotides (Pasqualini et al., 2009). Once active, MAPKs can phosphorylate many target molecules, both in the cytosol and in the nucleus, including enzymes or transcription factors (Rodriguez et al., 2010). The responses mediated by MAPKs involve induction of antioxidative or pro-oxidative enzymes and may attenuate or amplify the original signal triggered by ROS and NO (Asai et al., 2008; Opdenakker et al., 2012). In addition, MAPKs may also interfere in the signaling and in the biosynthesis of hormones, leading to the activation of downstream stress responses. The signal is interrupted by phosphatases, which promote the dephosphorylation of MAPK (Opdenakker et al., 2012).

Plant hormones are also critical messengers in plant development and stress tolerance. The signaling cascades of most hormones include interactions with ROS and NO, as is the case of ABA, mentioned above, and auxin. Auxin is an essential plant hormone that participates in various cellular processes. In certain situations, auxin induces the synthesis of ROS and NO, which, in turn, influence the hormone-mediated signaling (Joo et al., 2001; Yadav et al., 2013). ROS accumulation in stressful situations can trigger oxidative inactivation or degradation of auxin, as well as a decrease in the expression of genes involved in its transport and signaling, through specific MAPK cascades (Xia et al., 2015). GSNO accumulation also compromises the polar transport of auxin and reduces its effects via *S*-nitrosylation of components of its signaling pathway (Shi et al., 2015). The attenuation of auxin signaling leads to changes in plant growth and acclimatization to new environmental conditions. In some cases, however, ROS and NO can act as positive regulators of auxin (Woodward and Bartel, 2005; Terrile et al., 2012). Another hormone involved in the acclimation of plants to stress is salicylic acid (SA), which is responsible for transcriptional reprogramming during the defense against abiotic stress. The interaction between SA, ROS, and NO is complex, with ROS and NO acting both upstream and downstream of SA. NO and ROS induce the synthesis of SA, and NO also activates transcription factors that initiate SA-dependent gene expression by inducing the synthesis of various molecules, such as pro-oxidants and antioxidants (Mur et al., 2013). The synthesis of pro-oxidants and antioxidants during SA-mediated signaling presents biphasic redox dynamics. In the first phase, the oxidative phase, transient increases in ROS levels trigger signaling events that are dependent on the cell redox state, and this is followed by a reductive phase characterized by increased antioxidants and decreased ROS (Herrera-Vásquez et al., 2015). In addition to ABA, auxin, and SA, several other hormones, such as ethylene, gibberellins, and brassinosteroids, interact with ROS and NO in the process of acclimation to stress (Bartoli et al., 2013; Xia et al., 2015). These hormones also interact with each other and with other cellular messengers, such as Ca^{2+}, and the final response is dependent on the fine balance between all of these components (Xia et al., 2015).

In animals, NO-mediated signaling is largely dependent on the synthesis of cyclic nucleotides, especially cyclic GMP (cGMP). In these organisms, NO binding to a soluble guanylate cyclase increases the activity of this enzyme up to 200 times, with a consequent increase in the formation of cGMP (Francis et al., 2010). In plants, both NO and H_2O_2 increase the concentration of cyclic nucleotides, which apparently involves the binding of these molecules to enzymes with guanylate cyclase activity (Dubovskaya et al., 2011; Mulaudzi et al., 2011). In *Chlamydomonas*, an NO-dependent guanylate cyclase that participates in the transcriptional repression of NR has been identified, suggesting a model that integrates, among other components, NO, cGMP and the nitrogen assimilation pathways (de Montaigu et al., 2010). Two different types of responses are associated with cGMP, depending on the time between the perception of the stimulus and the peak in nucleotide concentration: fast responses, which involve the modulation of ion channels, such as Ca^{2+} channels; and long-term adaptive responses, which result in changes in the transcriptome and in the proteome (Pasqualini et al., 2009). In addition to increasing the concentration of cGMP (Durner et al., 1998; Dubovskaya et al., 2011), ROS and NO also promote nitration of this molecule, changing its structure and function, and the stomatal closure is one of the clearest examples of the interaction of NO, ROS, and cGMP (Joudoi et al., 2013).

Ca^{2+} is a highly versatile signaling molecule that plays a central role in the response to environmental stressors (Dodd et al., 2010; Schulz et al., 2013; Chmielowska-Bak et al., 2014). The exposure to stressors activates calcium channels, pumps and transporters in the plasma membrane or in the membranes of organelles, which results in the rapid influx of the cation into the cytosol, increasing the concentration of cytosolic Ca^{2+}. Transient calcium levels in the cytosol are detected by calcium-binding proteins such as calmodulin, Ca^{2+}-dependent protein kinases (CDPKs), phosphatases regulated by Ca^{2+} and by changes in Ca^{2+} channels and pumps (Gilroy et al., 2014), and these signals are then transmitted. One example is the Ca^{2+}-permeable channels in the plasma membrane, which are relatively inactive under normal conditions but can be activated by ROS in the guard cells in response to drought (Pei et al., 2000; Dodd et al., 2010). Similar to ROS, NO synthesis is induced by Ca^{2+}, and it activates intracellular Ca^{2+}-permeable channels and CDPKs (Astier et al., 2010).

FUTURE PERSPECTIVES

It is widely recognized that ROS and NO interact with each other and are key molecules in the plant response to various types of abiotic stresses. Additionally, a large body of evidence has shown that cellular redox signaling contributes to the development of SAA in plants and, in some cases, priming, which can involve other signaling networks, such as hormones and MAPK cascades. However, although there has been significant

progress in elucidating the interplay between ROS and NO, many challenges remain. There is still little information, for example, on the initiation of the signaling mediated by ROS and NO, the mechanisms involved in the perception and the specificity of the generated signal, and the control of the delicate balance between production and scavenging of the reactive species. Detailed studies investigating cross-talk regulation among ROS, NO, hormones, cyclic nucleotides, MAPKs, and other signaling molecules are needed to clarify how these molecules interact with each other during different types of stresses.

Data obtained in recent years have shown that both ROS and NO trigger post-translational modifications of proteins, an important process for signal transduction. In fact, recent research analyzed the impact of ROS and NO on the *S*-nitroso-proteome, or redox proteome, of plants exposed to various stressors. In addition to these studies, further biochemical and functional characterizations of proteins that have been post-translationally modified are needed to provide a more comprehensive understanding of the effects of ROS and NO at the molecular level. It is also necessary to evaluate in more detail how these signaling molecules alter gene expression by analyzing, for example, their possible involvement in epigenetic processes. Finally, it is important to note here that many studies examining ROS and NO signaling used exogenous sources of NO or substances that induce the generation of H_2O_2. Despite the clear relevance of these studies, it is important to analyze ROS and NO naturally produced in response to the environment, thus contributing to understanding the signaling process *in planta*.

AUTHOR CONTRIBUTIONS

FF outlined the manuscript together with JO and wrote the following topics: biosynthesis, metabolism, and transport; molecular bases of ROS and NO action; and perspectives. PM-S wrote all the part about the different kinds of abiotic stresses (drought, hypoxia, salt stress, thermal stress, and heavy metal). GG wrote the introduction and the topic "Interaction among ROS, NO, and Other Signaling Molecules." JO outlined the manuscript together with FF, guided and led the discussions and made a critical review of all the manuscript.

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Nitric Oxide Alleviates Salt Stress Inhibited Photosynthetic Performance by Interacting with Sulfur Assimilation in Mustard

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The role of nitric oxide (NO) and sulfur (S) on stomatal responses and photosynthetic performance was studied in mustard (*Brassica juncea* L.) in presence or absence of salt stress. The combined application of 100 μM NO (as sodium nitroprusside) and 200 mg S kg^{-1} soil (S) more prominently influenced stomatal behavior, photosynthetic and growth performance both in the absence and presence of salt stress. The chloroplasts from salt-stressed plants had disorganized chloroplast thylakoids, but combined application of NO and S resulted in well-developed chloroplast thylakoids and properly stacked grana. The leaves from plants receiving NO plus S exhibited lower superoxide ion accumulation under salt stress than the plants receiving NO or S. These plants also exhibited increased activity of ATP-sulfurylase (ATPS), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) and optimized NO generation that helped in minimizing oxidative stress. The enhanced S-assimilation of these plants receiving NO plus S resulted in increased production of cysteine (Cys) and reduced glutathione (GSH). These findings indicated that NO influenced photosynthesis under salt stress by regulating oxidative stress and its effects on S-assimilation, an antioxidant system and NO generation. The results suggest that NO improves photosynthetic performance of plants grown under salt stress more effectively when plants received S.

Keywords: antioxidant, nitric oxide, photosynthesis, salt stress, sulfur

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INTRODUCTION

The global aim of increasing agricultural productivity by 70% by the year 2050 for approximately 2.3 billion individuals is facing severe obstructions, primarily due to increasing abiotic stress factors (FAO, Food and Agricultural Organization, 2009). These factors such as cold, drought, flooding, freezing, heat, salinity, or oxidizing agents disturb plant metabolism and negatively impact productivity (Wang et al., 2004; Mian et al., 2011). Salt stress is one of the major abiotic stress factors occupying more than 45 million hectares of irrigated land (Munns and Tester, 2008). It causes excess production of reactive oxygen species (ROS) resulting in induced oxidative stress and inhibition of the Calvin-Benson cycle enzymes (Fatma et al., 2014; Nazar et al., 2014). Plants growing under salt stress develop detoxification mechanisms to avoid damage induced by ROS. These mechanisms are upregulation of activity of enzymatic antioxidants; ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), and superoxide dismutase, and production of non-enzymatic antioxidants; ascorbate (AsA), reduced glutathione (GSH), carotenoids, tocopherol,

that help in neutralizing or scavenging ROS (Noctor et al., 2012; Khan and Khan, 2014; Nazar et al., 2015).

Sulfur (S) is the fourth major essential nutrient element with important roles in stress tolerance of plants (Marschner, 1995; Iqbal et al., 2013). Khan et al. (2013) have reported that S is a constituent of major metabolic compounds, such as cysteine (Cys), methionine, GSH, proteins, coenzyme A, sulfo-lipids, iron-sulfur (Fe-S) clusters, glucosinolates, vitamins (biotin and thiamine), and thioredoxin system that have the potential to modulate the physiological processes of plants to alleviate the negative effect of salt stress (Khan and Khan, 2014). Sulfur plays a significant role in the build up of photosynthetic apparatus and electron transport system (Marschner, 1995). The deficiency of S impairs plant metabolism (Honsel et al., 2012) and reduces the chlorophyll content and photosynthesis in *Beta vulgaris* (Kastori et al., 2000). Moreover, its deficiency decreases the photosynthetic efficiency by affecting the content and activity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in *Oryza sativa* (Lunde et al., 2008).

The GSH production is correlated with S-assimilation as both were found up-regulated under conditions of oxidative stress (Noctor et al., 2012). It has been reported that the rate of S-assimilation and GSH biosynthesis were greatly increased in *Brassica napus* plants exposed to saline conditions (Ruiz and Blumwald, 2002), and the increased S supply helped in the protection of *Hordeum vulgare* plants from salt induced-oxidative stress by increasing GSH content (Astolfi and Zuchi, 2013). The exogenous supplementation of GSH in *B. juncea* improved the cell redox state (GSH/GSSG) for better protection and adaptation against salt stress and improvement of photosynthetic capacity (Fatma et al., 2014). Kopriva and Rennenberg (2004) have reported that GSH acted as a signal molecule for S status of plants and was sensitive to ATP-sulfurylase (ATPS), the first enzyme in S-assimilatory pathway (Yi et al., 2010). While Szalai et al. (2009) observed the essential role for GSH in stress tolerance, Mckersie and Leshem (1994) and Gondim et al. (2013) explained that GSH reacts with $^1O_2$, $O_2^{\cdot -}$, and $^{\cdot}OH$ ions and functions as a free radical scavenger.

Phytohormones are known to alleviate salt stress by regulating S-assimilation in plants (Fatma et al., 2013). In particular, nitric oxide (NO) has recently been regarded as a potential plant hormone related to plant defense reactions (Gould et al., 2003). The exogenous application of NO improved salt tolerance by alleviating the oxidative damage (Beligni and Lamattina, 2000), stimulating activity of proton-pump and Na^+/H^+ antiport in the tonoplast and increasing the K^+/Na^+ ratio (Beligni and Lamattina, 2002). It has been reported in *Sorghum bicolor* that NO controls activity of phosphoenolpyruvate carboxylase kinase, and mediates responses of plants to salt stress (Monreal et al., 2013). The improvement of photosynthesis after NO application was due to quenching of excess energy and increase in quantum yield of PSII in *Solanum melongena* seedlings (Wu et al., 2013). Recently, we have shown that NO application enhanced the photosynthetic potential of *B. juncea* under salt stress (Fatma and Khan, 2014). In the presence of stress, NO combines with GSH and forms S-nitrosoglutathione (GSNO) resulting in enhanced S requirement of plants for better survival (Wang et al., 2015a). Studies of Xiong et al. (2010) and Wang et al. (2015b) have shown that NO enhanced tolerance against oxidative stress induced by metals by stimulating GSH biosynthetic pathway.

It is evident from the available literature that the individual application of NO or S improves salt stress tolerance in plants. The alleviation of salt stress with S supplementation involves GSH production, but how NO alleviates salt stress in plants receiving S is not clear. It was, therefore, hypothesized that application of NO in presence of S improved S-assimilation, GSH production, and modulated NO generation to counteract the adverse effects of salt stress on photosynthetic performance of plants. The objectives of the reported research was to study S-assimilation, the antioxidant system and NO generation influenced by NO and S application, and to find out the extent to which photosynthetic performance of salt grown plants was improved.

MATERIALS AND METHODS

Plant Material, Growth Conditions and Treatments

Healthy seeds of mustard (*B. juncea* L. Czern & Coss. var. Varuna) were surface sterilized with 0.01% $HgCl_2$ followed by repeated washings with distilled water and were sown in 23-cm diameter earthen pots containing 5 kg soil with peat and compost (4:1, w/w) mixed with sand (3:1, w/w). The pots were kept under natural day/night conditions with photosynthetically active radiation ~640 μmol m^{-2} s^{-1}, average day/night temperature of 22/14 ± 3°C and relative humidity 62–70% in a net house of the Botany Department, Aligarh Muslim University, Aligarh, India. In the experiment, elemental S was used for obtaining 200 mg S kg^{-1} soil (S) by applying 10 days before sowing. Our earlier research has shown that 200 mg S kg^{-1} soil and 100 mg S kg^{-1} soil are excess-S and sufficient-S, respectively, and excess-S promoted photosynthesis and growth more than sufficient-S in the presence of salt through the GSH production (Fatma et al., 2014). NaCl at 100 mM was added to soil before seed sowing for creating salt stress. The addition of 100 mM NaCl develops 10.0 dS m^{-1} salinity (Khan et al., 2009). One hundred ml of NaCl or water was given alternately for 15 days from the sowing time. The concentration of NaCl was selected based on our earlier findings (Fatma et al., 2014). The native soil S concentration was 100 mg S kg^{-1} soil. A concentration of 100 μM NO (as sodium nitroprusside) was applied on foliage of plants alone or on S-grown plants in presence or absence of NaCl with a hand sprayer at 20 days after sowing (DAS). A surfactant teepol (0.5%) was added with the control and NO treatments. Treatments were arranged in a complete randomized block design and number of replicates for each treatment was four ($n = 4$).

Measurements

Chlorophyll Content

SPAD chlorophyll meter (SPAD 502 DL PLUS, Spectrum Technologies) was used for the expression of the values of chlorophyll content.

Photosynthetic Gas Exchange

Net photosynthesis (P_N), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were measured between 11.00 and 12.00 at light saturating intensity on a sunny day in fully expanded uppermost second leaves of plants in each treatment using Infra Red Gas Analyzer (CID-340, Photosynthesis system, Bio-Science, USA). The atmospheric conditions at the time of measurement were: photosynthetically active radiation, ∼680 μmol m^{-2} s^{-1}; air temperature, ∼22°C and relative humidity, ∼70%.

PS II Activity

Chlorophyll fluorometer (Junior-PAM, Heinz Walz, Germany) was used for determining the maximal PS II photochemical efficiency (Fv/Fm) of the fully expanded second leaf from top of the plant. Minimal fluorescence (Fo) and maximal fluorescence (Fm) were obtained by dark adapting the plants for 30 min. The Fo was measured during the weak measuring pulses (125 μmol m^{-2} s^{-1}) and a saturating pulse (720 μmol $m^{-2}s^{-1}$) was used to obtain Fm. Variable fluorescence (Fv) was estimated by the difference between Fo and Fm. The quantum yield efficiency of PS II was represented by the ratio of variable fluorescence to maximal fluorescence.

Rubisco Activity

Rubisco activity was determined by monitoring NADH oxidation at 30°C at 340 nm during the conversion of 3-phosphoglycerate to glycerol 3-phosphate after the addition of enzyme extract to the assay medium (Usuda, 1985). Leaf tissue (1 g) was homogenized in a chilled mortar and pestle with ice-cold extraction buffer that contained 0.25 M Tris-HCl (pH 7.8), 0.05 M $MgCl_2$, 0.0025 M EDTA, and 37.5 mg DTT for enzyme extraction. The centrifugation of homogenate was done at 10,000 × g for 10 min at 4°C. The supernatant was used to assay the enzyme. The reaction mixture included 100 mM Tris-HCl (pH 8.0), 40 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.2 mM NADH, 4 mM ATP, 0.2 mM EDTA, 5 mM DTT, 1 U of glyceraldehyde-3-phosphodehydrogenase and 1 U of 3-phosphoglycerate kinase and 0.2 mM ribulose 1,5-bisphosphate.

SDS-PAGE for Rubisco

Protein extraction was done by adopting the method of Carvalho et al. (2005) with slight modifications and Bradford (1976) method was used for the estimation of protein content using bovine serum albumin as standard. Fresh leaf of the plant was taken for the extraction of protein for gel electrophoresis. Leaf tissues (500 mg) were homogenized in extraction buffer (potassium phosphate buffer 0.2 M, pH 8.0) containing: 5 mM EDTA, 5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride and 50% (w/w) polyvinylpyrrolidone with a small amount of quartz sand. The extracts were centrifuged at 27,000 × g for 10 min at 4°C, and the resulting supernatant was desalted through sephadex PD10 columns Amersham Biosciences equilibrated through the extraction buffer (50 mM, pH 7.5). For all subsequent analyses, the desalted extracts stored at 20°C were used as the source of total soluble protein. Stacking gel of 5% (w/v) and 12.5% in the separation gel (w/v) were used for resolving the protein samples and stained with Coomassie Brilliant Blue R-250 (Sigma) (Laemmili, 1970). Equal amount of 40 μg proteins were loaded onto SDS-PAGE wells uniformly. Molecular weights of protein band were determined in respect to standard protein marker (Merck, pre stained 14.3–97.4 kDa). ImageJ software was used to compare the density/intensity of bands on gel. The density of the bands was presented as relative to the control band.

Biochemical Analyses

Determination of Na^+ and Cl^- Ions

Root and leaf samples were washed to determine content of Na^+ and Cl^-. Plant samples (500 mg) were digested in 19 ml Tri acid mixture, containing 10 ml of 16 M HNO_3, 5 ml of 18 M H_2SO_4 and 4 ml of 11.65 M $HClO_4$ in the ratio of 10:5:4 (v/v). The content of ions was extracted in distilled water by boiling for 30 min twice. The filtered extract was used to measure Na^+ using flame photometer (Khera-391: Khera Instruments, New Delhi), and Cl^- content was determined by titrating against 0.02 N $AgNO_3$ solution using 5% K_2CrO_4 as the indicator.

Determination of H_2O_2 Content and Lipid Peroxidation

The details of the determination of content of H_2O_2 have been given in Fatma et al. (2014). Briefly, the content of H_2O_2 was determined by adopting the method of Okuda et al. (1991) in leaf tissues (500 mg) grounded in ice-cold 200 mM $HClO_4$. This was centrifuged at 1200 × g for10 min followed by neutralization of $HClO_4$ of the supernatant with 4 M KOH. The insoluble $KClO_4$ was eliminated by further centrifugation at 500 × g for 3 min. In a final volume of 1.5 ml, the reaction mixture contained 1 ml of the eluate, 400 μl of 12.5 mM 3-(dimethylamino) benzoic acid in 0.375 M phosphate buffer (pH 6.5), 80 μl of 3-methyl-2-benzothiazoline hydrazone and 20 μl of peroxidase (0.25 Unit). The reaction was started by the addition of peroxidase at 25°C and the increase in absorbance was recorded at 590 nm.

Lipid peroxidation was expressed as the content of thiobarbituric acid reactive substances (TBARS) and was estimated using the method of Dhindsa et al. (1981). Leaf tissues (500 mg) were ground in 0.25% 2-thiobarbituric acid in 10% trichloroacetic acid and the mixture was heated at 95°C for 30 min and cooled quickly on ice bath. This was followed by centrifugation at 10,000 × g for 10 min. Four ml of 20% trichloroacetic acid containing 5% thiobarbituric acid was added to 1 ml aliquot of the supernatant. The intensity of the color was read at 532 nm.

Accumulation of Superoxide Ion

The accumulation of superoxide ion (O_2^-) in leaves was noted by histochemical staining method by adopting the method of Wang et al. (2011) with slight modification using nitro blue tetrazolium (NBT). The samples (3 leaves) from each treatment were immersed into 1 mg/ml NBT solution prepared in 10 mM phosphate buffer (pH 7.8) at ambient temperature (23°C) under day light for 6 h. The blue spots on NBT staining appeared, which were cleared in concentrated ethanol and then kept in 70% ethanol. The pictures were taken with a NIKON digital camera (COOLPIX110).

Antioxidant Metabolism

Assay of Antioxidant Enzymes

The method of Aebi (1984), Nakano and Asada (1981), and Foyer and Halliwell (1976) with slight modifications, were adopted for the measurement of CAT, APX, and GR activity, respectively. Fresh leaf tissues (200 mg) were homogenized in chilled mortar and pestle with an extraction buffer containing 0.05% (v/v) Triton X-100 and 1% (w/v) polyvinylpyrrolidone in 100 mM potassium-phosphate buffer (pH 7.0). At 4°C, the homogenate was centrifuged at 15,000 × g for 20 min. The supernatant obtained after centrifugation was used for the assay of CAT (EC; 1.11.1.6) and GR (EC; 1.6.4.2). For the assay of APX (EC; 1.11.1.11) extraction buffer was supplemented with 2 mM AsA. The details of procedure have been described earlier in our studies (Fatma et al., 2014).

S-assimilation

Activity of ATPS and Cys Content

The method of Lappartient and Touraine (1996) was adopted for the measurement of ATPS activity. The activity of ATPS was assayed *in vitro* in leaves by measuring molybdate-dependent formation of pyrophosphate.

Content of Cys in leaves was determined spectrophotometrically adopting the method of Gaitonde (1967). The amount of Cys was calculated with reference to a calibration curve obtained under similar conditions for standard Cys covering a range of 5–20 nmol. The details of the determination have been described earlier (Fatma et al., 2014).

S Content

Oven-dried leaf powder (100 mg) was taken in 75 ml digestion tube. In the tube, 4 ml acid mixture (contained concentrated HNO_3 and $HClO_4$ in the ratio of 85:1, v/v) and 7.5 mg of selenium dioxide as catalyst were added. The mixture was digested and the volume of the colorless solution was made up to 75 ml with deionized water. The interference of silica was checked by filtering the contents of the tube. A 5 ml aliquot was pipette out from the digested solution for turbidity development in 25 ml volumetric flask. Turbidity was developed by adding 2.5 ml gum acacia (0.25%) solution, 1.0 g $BaCl_2$ sieved through 40–60 mm mesh and the volume was made up to the mark with deionized water. The contents of 25 ml volumetric flask were thoroughly shaken till $BaCl_2$ completely dissolved. Turbidity was allowed to develop for 2 min. The values were recorded at 415 nm within 10 min after the turbidity development. A blank was also run simultaneously after each set of determination. The amount of sulfate was calculated with the help of a calibration curve drawn a fresh using a series of K_2SO_4 solutions. The method has been given earlier (Fatma et al., 2014).

GSH Content and Redox State

The determination of GSH was done using the method of Griffith (1980). Reduced glutathione was assayed by an enzyme recycling procedure in which it was sequentially oxidized by 5, 5′-dithiobis-2-nitrobenzoic acid and reduced by NADPH in the presence of GR. GSH was masked by derivatization with 2-vinylpyridine for the assay of GSSG. Fresh leaf tissues (500 mg) were ground in liquid nitrogen using mortar and pestle and suspended in 2 ml of 5% (w:v) sulfosalicylic acid and then centrifuged at 12,000 × g for 10 min. The details for the determination of GSH and redox state have been elaborated in Fatma et al. (2014).

NO Generation

Generation of NO was determined by estimating nitrite content adopting the method previously described by Zhou et al. (2005) with minor modifications. Homogenization of leaves (500 mg) was done in 3 ml of 50 mM ice cold acetic acid buffer (pH 3.6) containing 4% zinc acetate using mortar and pestle. Thereafter, the homogenate was centrifuged at 11,500 × g for 15 min at 4°C and the supernatant was collected. The pellet was washed with 1 ml of the extraction buffer and again centrifuged. Supernatants from the two spin were combined and neutralized by adding 100 mg of charcoal. The filtrate was leached and collected, after vortex and filtration. One ml each of the filtrate and Greiss reagent (1% sulphanilamide and 0.1% N-1-napthylethylenediaminedihydrochloride in 5% H_2PO_4 solution) mixed in the ratio (1:1) was incubated at room temperature for 30 min. Finally, the absorbance of the reaction mixture was taken at 540 nm and NO content was estimated from a calibration curve plotted using sodium nitrite as standard.

Abscisic Acid Determination

The content of abscisic acid (ABA) was determined according to the method previously described by Hung and Kao (2003) with slight modifications. Leaves were frozen with liquid nitrogen immediately and ground into fine powder. Then, the powder was homogenized in the extraction solution (80% methanol containing 2% glacial acetic acid) using a motor and pestle. The crude extract was centrifuged and passed through polyvinylpyrrolidone column and C_{18} cartridges to remove plant pigments and other non-polar compounds which could interfere in the immunoassay. The eluates were then concentrated to dryness by vacuum evaporation and resuspended in Tris-buffered saline before enzyme-linked immunosorbent assay (ELISA). Subsequently, ABA was determined with ABA immunoassay detection kit (PGR-1; Sigma-Aldrich, St. Louis, MO, USA) as per the user manual. The values were recorded at 405 nm and the ABA content was estimated from a calibration curve plotted by using standard ABA.

Structural and Chloroplast Ultrastructure Studies

Confocal Microscopy

For confocal microscopy young axillary leaves of plant was plucked and dried in a desiccator. Thereafter, the leaves were processed from the dorsal side to remove the epidermal layer and expose the stoma. The leaves were fine sectioned and mounted on glycerol cover-slip on glass slides. The samples were then analyzed under the Olympus Fluoview TM-FV1000 confocal microscope at 60X magnification and 1X optical zoom. DIC images were captured for different samples keeping fixed microscopy parameters. Fluoview FV10 ver 1.7 was used to analyze samples and scale bars were used.

Scanning Electron Microscopy

Leaf samples were prepared for scanning electron microscopy (SEM) by adopting the method of Daud et al. (2009) with slight modifications. Fresh leaves samples were taken from the axillary positions (ideally the leaf was 1.5 × 1.5 inch in size) and were preferably air dried in dessicator. Subsequently, leaf samples were first fixed with 2.5% glutaraldehyde plus 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) in equal quantity for more than 4 h and then washed three times with phosphate buffer for 15 min at each step. The samples were then post fixed with 1% osmium oxide in phosphate buffer (pH 7.0) for 1 h and washed three times with the same phosphate buffer for 15 min. After that, firstly, the specimens were dehydrated by a graded series of ethanol (50, 70, 80,90, 95, and 100%) for about 15–20 min at each step, transferred to the mixture of alcohol and iso-amyl acetate (v/v = 1) for about 30 min. Then, the samples were transferred to pure iso-amyl acetate for 1 h. In the end, the specimens were dehydrated in Carl Zeiss EVO 40 (Germany) scanning electron microscope critical point dryer with liquid CO_2. The dehydrated specimen was coated with gold-palladium and observed under the Carl Zeiss EVO 40 (Germany) scanning electron microscope at extra high tension or high voltage at 20 kV and magnification of 1500 X or 5000 X. The stomata were observed under the scanning electron microscope at 1.50 K X and 5.0 K X. The stomatal frequency was determined by counting the number of stomata in the microscope field of view.

Transmission Electron Microscopy

Leaf tissues for chloroplast ultrastructure were prepared for transmission electron microscopy (TEM) by adopting the method of Sandalio et al. (2001) with slight modifications. Leaf samples were cut with razor blade into 1 mm^2 segments and fixed in 2.5% glutaraldehyde solution in 50 mM phosphate buffer (pH 6.8) for 2.5 h at room temperature. Leaf tissue was then post-fixed for 30 min in 1% osmium tetroxide in 50 mM sodium cacodylate buffer (pH 7.2) and dehydrated in ethanol graded series (30–100%, v/v). After dehydration in a graded series of ethanol, replaced to propylene oxide, and then the tissue was embedded in Spurr resin. Ultrathin sections were taken by using Leica EM UC6 ultramicrotome. Sections were stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (JEOL 2100F, JAPAN) accelerating voltage at 120 kV and magnification at 6000 X and 1200 X. The chloroplast ultrastructure (thylakoid membranes) was observed from TEM images.

Growth Characteristics

From each pot, plants were uprooted carefully and washed to remove dust. Leaf area was measured with the help of leaf area meter (LA 211, Systronics, New Delhi, India).

After drying the sample in a hot air oven at 80°C till constant weight, the plants were recorded for obtaining dry mass.

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 $P < 0.05$. Bars showing the same letter are not significantly different by LSD test at $P < 0.05$.

RESULTS

Effect of NO and S on Photosynthetic Performance

The plants receiving NO or S showed higher values for P_N, g_s, C_i, PSII activity and Rubisco activity compared to control plants in the absence or presence of salt. Application of NO or S in plants without salt equally increased P_N by 66.0%, g_s by 31.0%, C_i by 38.0%, maximal PS II photochemical efficiency by 18.0% and Rubisco activity by 38.0% in comparison to control plants. In presence of salt, NO or S increased P_N, g_s, C_i, maximal PS II photochemical efficiency and Rubisco activity by 12.1% or 35.6%, 9.6% or 21.6%, 12.5% or 25.9%, 5.5% or 12.3%, and 11.1% or 20.8%, respectively compared to control plants (**Table 1**). Application of NO and S together resulted in maximum values of the above observed photosynthetic parameters in plants grown with or without salt compared to control. In the presence of salt, plants receiving NO and S together alleviated the effects of salt stress and enhanced P_N, g_s, Ci, maximal PS II photochemical efficiency and Rubisco activity by 80.3, 47.1, 52.2, 24.7, and 72.2%, respectively compared to control plants (**Table 1**).

SDS-PAGE for Rubisco

SDS-PAGE showed differences in the intensity of Rubisco protein band of plants grown with salt or plants treated with NO and S in absence or presence of salt (**Figure 1**). Salt treatment resulted in degradation of protein and thus low intensity of protein band. On the other hand, plants receiving NO or S exhibited distinct protein band, but maximal increase in the density/intensity of protein band was obtained with the combined treatment of NO plus S. The effect of S was found more pronounced than NO in increasing the protein band intensity (**Figure 1**). The relative

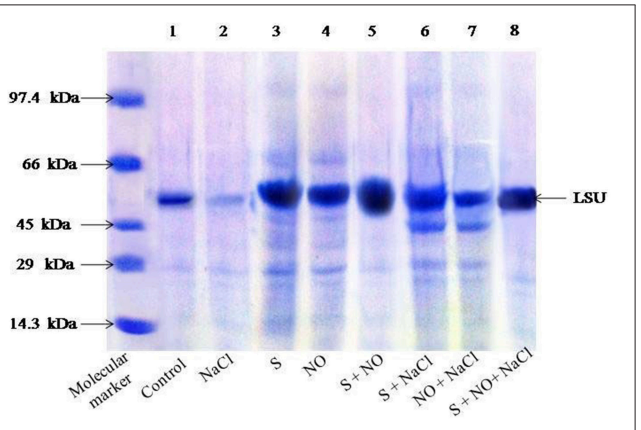

FIGURE 1 | SDS-PAGE protein profile of Rubisco of mustard *(Brassica juncea* L.) leaves treated with 100 μM nitric oxide (NO) and grown with S (200 mg S kg^{-1} soil; S) in presence or absence of 100 mM NaCl at 30 DAS. Equal amount of protein (40 μl) were loaded on to each lane. The proteins expressed in the range of 14.3–97.4 kDa. LSU, large subunits of Rubisco (native soil S content: 100 mg S kg^{-1} soil).

TABLE 1 | Chlorophyll content, maximal PS II photochemical efficiency, Rubisco activity, stomatal conductance, intercellular CO_2 concentration, and net photosynthesis of mustard (*Brassica juncea* L.) leaves treated with 100 μM nitric oxide (NO) and/or grown with S (200 mg S kg^{-1} soil; S) in presence or absence of 100 mM NaCl at 30 DAS.

Treatments	Chlorophyll content (SPAD value)	Maximal PS II photochemical efficiency	Rubisco activity (μmol CO_2 mg^{-1} protein min^{-1})	Stomatal conductance (mmol CO_2 m^{-2} s^{-1})	Intercellular CO_2 concentration (μmol CO_2 mol^{-1})	Net photosynthesis (μmol CO_2 m^{-2} s^{-1})
0	32.7 ± 1.18^{f}	0.73 ± 0.015^{f}	0.72 ± 0.030^{f}	365 ± 11.1^{f}	255 ± 10.4^{f}	13.2 ± 0.61^{f}
NaCl	22.7 ± 1.11^{g}	0.58 ± 0.014^{g}	0.43 ± 0.028^{g}	280 ± 09.3^{g}	173 ± 07.3^{g}	7.8 ± 0.55^{g}
S	42.1 ± 1.62^{c}	0.87 ± 0.018^{c}	1.02 ± 0.051^{c}	483 ± 18.7^{c}	360 ± 14.7^{c}	22.2 ± 0.75^{c}
NO	41.2 ± 1.60^{c}	0.86 ± 0.018^{c}	0.99 ± 0.047^{c}	479 ± 18.5^{c}	352 ± 14.1^{c}	21.9 ± 0.71^{c}
S + NO	50.3 ± 1.85^{a}	0.97 ± 0.019^{a}	1.37 ± 0.039^{a}	578 ± 22.3^{a}	428 ± 15.5^{a}	26.5 ± 0.90^{a}
S + NaCl	38.0 ± 1.55^{d}	0.82 ± 0.018^{d}	0.87 ± 0.040^{d}	444 ± 17.0^{d}	321 ± 13.7^{d}	17.9 ± 0.68^{d}
NO + NaCl	35.0 ± 1.45^{e}	0.77 ± 0.017^{e}	0.80 ± 0.030^{e}	400 ± 14.0^{e}	287 ± 11.9^{e}	14.8 ± 0.67^{e}
S+ NO+ NaCl	46.2 ± 1.70^{b}	0.91 ± 0.019^{b}	1.24 ± 0.035^{b}	537 ± 20.8^{b}	388 ± 15.1^{b}	23.8 ± 0.81^{b}

Data are presented as treatments mean ± SE (n = 4). Data followed by same letter are not significantly different by LSD test at $P < 0.05$. Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

density of all bands was calculated with respect to control. The density of bands decreased by 45% under salt stress, while the density of bands increased by 31 and 23%, respectively with S or NO in comparison to control. The combined treatment of NO and S increased the density of bands maximally by 43% in comparison to control in absence of salt. In presence of salt, S and NO increased the band density by 34% in comparison to control.

Effect of NO and S on Oxidative Stress

The content of Na^+ and Cl^- increased in roots and leaves in salt treated plants (**Table 2**). Application of NO or S resulted in reduction of Na^+ and Cl^- content in both roots and leaves under no stress compared to control plants. Under salt stress, S application proved better than NO in lowering content of Na^+ and Cl^- in comparison to control plants. However, the reductions in the content of ions were greatest when plants were supplemented with NO plus S under both salt stress and no stress conditions.

Salt stress increased content of H_2O_2 and TBARS equally by about two-times compared to control plants. The individual application of S and NO reduced the content of H_2O_2 and TBARS by about 40 and 30% under no stress, but these reductions were about 24 and 12%, respectively under salt stress compared to control plants. The maximal reduction in content of H_2O_2 and TBARS was found with the combined treatment of NO and S under no stress and salt stress in comparison to control plants (**Table 2**).

Histochemical staining with NBT to demonstrate the O_2^- accumulation showed that leaves were scarcely stained by NBT in the absence of dehydration (**Figure 2**), but were blue stained (marker for O_2^- accumulation) when the leaves were exposed to dehydration for 6 h. The leaves from the salt treated plants showed deepest staining, while NO plus S treatment resulted in least staining compared to control plants. NO or S treatment resulted in shallow staining compared to control plants. Under salt stress, plants treated with NO plus S showed lesser staining than their individual treatments and salt treated plants. The effect of S was more prominent in reducing the accumulation of O_2^- than NO in presence of salt.

Effect of NO and S on Antioxidant Enzymes

The activity of CAT, APX, and GR was assayed in order to evaluate the extent to which the antioxidant enzymes system was influenced by salt stress and how they were modulated by NO and S. Salt stress increased the activity of these enzymes compared to control plants. NO or S increased the activity of CAT and GR equally by about 73%, and APX activity by about 2.5-times under no stress compared to control plants. In salt grown plants, NO and S individually increased the activity of CAT by 65 and 40.8%, APX by 96.6 and 73.9% and GR by 63.1 and 47.3%, respectively in comparison to control plants. However, maximum increase in the activity of these enzymes was noted with the combined treatment of NO and S under both no stress and salt stress conditions (**Figures 3A–C**).

Effect of NO and S on S-assimilation and Redox State (GSH/GGSG)

The influence of NO or S was assessed on S-assimilation to monitor its contribution in salt stress alleviation. Salt stress decreased S content (−14.2%) and redox state (−56.8%), but increased the activity of ATPS (+13.3%) and accumulation of Cys (+15.2%) and GSH (+21%) in comparison to control. The decrease in S content and redox state by salt stress was recovered by the application of NO or S almost equally by 50% compared to control plants. Moreover, application of NO or S also equally increased the activity of ATPS by 83%, Cys content by 55% and GSH content by 70% compared to control plants under no stress. In salt stressed plant, GSH content was higher than the control plants suggesting that plants used the available S for GSH synthesis resulting in lower values of S content. Plants treated with S under salt stress exhibited increased ATPS activity (+61.6%), Cys content (+45.7%), S (+24.4%), GSH content (+49.1%), and redox state (+25.7%) in comparison to control plants. However, a lesser increase in ATPS activity (+47.5%), Cys content (+28.8%), S content (+12.2%), and GSH content

TABLE 2 | Root and leaf Na^+ and Cl^- content and H_2O_2 and TBARS content in mustard (*Brassica juncea* L.) leaves treated with 100 μM nitric oxide (NO) and/or grown with S (200 mg S kg^{-1} soil; S) in presence or absence of 100 mM NaCl at 30 DAS.

Treatments	Root Na^+	Root Cl^-	Leaf Na^+	Leaf Cl^-	H_2O_2	TBARS
	(mg g^{-1} leaf dry mass)				(nmol g^{-1} leaf dry mass)	
0	8.80 ± 0.808^{e}	7.5 ± 0.340^{e}	8.9 ± 0.612^{e}	7.2 ± 0.303^{e}	18.7 ± 0.989^{b}	5.00 ± 0.220^{b}
NaCl	27.30 ± 0.412^{a}	18.1 ± 0.430^{a}	22.5 ± 1.000^{a}	14.9 ± 0.786^{a}	33.6 ± 0.988^{a}	10.10 ± 0.234^{a}
S	7.60 ± 1.077^{f}	5.5 ± 0.306^{f}	6.9 ± 0.532^{f}	5.2 ± 0.410^{f}	10.4 ± 1.020^{e}	3.10 ± 0.180^{e}
NO	7.80 ± 1.064^{f}	5.7 ± 0.309^{f}	7.3 ± 0.661^{f}	$5.5 \pm 0.444+$	11.2 ± 1.090^{e}	3.30 ± 0.190^{e}
S + NO	6.20 ± 0.602^{g}	4.2 ± 0.288^{g}	5.4 ± 0.560^{g}	4.2 ± 0.209^{g}	6.1 ± 0.870^{g}	1.93 ± 0.140^{g}
S + NaCl	13.70 ± 0.434^{c}	11.3 ± 0.555^{c}	13.8 ± 0.888^{c}	9.8 ± 0.530^{c}	14.2 ± 1.010^{d}	3.80 ± 0.190^{d}
NO + NaCl	15.20 ± 0.596^{b}	12.2 ± 0.612^{b}	16.5 ± 0.987^{b}	12.5 ± 0.612^{b}	16.2 ± 1.090^{c}	4.40 ± 0.208^{c}
S + NO+ NaCl	11.10 ± 0.707^{d}	8.8 ± 0.303^{d}	10.9 ± 0.732^{d}	8.5 ± 0.660^{d}	8.0 ± 0.920^{f}	2.80 ± 0.170^{f}

Data are presented as treatments mean ± SE (n =4). Data followed by same letter are not significantly different by LSD test at $P < 0.05$. Cl^-, chloride ion; H_2O_2, hydrogen peroxide; Na^+, sodium ion; TBARS, thiobarbituric acid reactive substances.

FIGURE 2 | *In situ* accumulation of superoxide ion (O_2^-) by nitro blue tetrazolium (NBT) staining of mustard (*Brassica juncea* L.) leaves after dehydration. The leaves originated from plants treated with/without 100 mM NaCl, S (200 mg S kg^{-1} soil; S) or 100 μM nitric oxide (NO) individually or in combinations at 30 DAS. Arrow (→) shows O_2^- accumulation.

(+36.8%) and redox state (+11.6%) was noted with NO in salt treated plants. Nevertheless, supplementation of NO plus S to plants grown with salt or no stress condition resulted in maximum increase in ATPS activity (+158.3 and +200%), Cys content (+72.8 and +84.7%), S (+71.4 and +79.5%), GSH (+87.7 and +96.4%) and redox state (+88.3 and +97.9%), respectively compared to control plants (**Table 3**).

Effect of NO and S on NO Generation

Plants grown with salt showed increased NO generation by 3.8-times compared to control plants, but exogenously applied NO resulted in reduced NO generation by 2.2-times compared to salt treated plants. However, maximum reduction in NO generation by 3-times was noted with S application compared to salt treated plants under no stress. The combined application of NO plus S decreased NO formation by 2.2-times compared to salt treated plants. Under salt stress, plants receiving NO or S reduced NO generation by 1.5 or 2-times, respectively compared to salt treated plants. The combination of NO and S in the presence of salt reduced NO generation by 1.7-times compared to salt-treated plants (**Figure 3D**).

Effect of NO and S on ABA Content

The content of ABA was influenced by NO and S given alone or in combination. Supplementation of plants with NO resulted in 1.7-times greater ABA accumulation than control plants. Plants given S treatment showed 2.7-times lesser ABA content compared to control plants. The combined application of NO and S also decreased ABA content compared to salt treated plants under no stress. Plants receiving NO or S under salt stress exhibited reduced ABA content by 1.3 or 1.6-times, respectively compared to salt treated plants. In salt treated plants, NO plus S decreased the ABA content by 1.4-times compared to salt treated plants (**Figure 4**).

Effect of NO and S on Stomatal Response

Stomatal Response

Confocal and electron microscopy were used to study the changes in guard cells in response to NO and S. Stomatal opening was 2 μm in diameter in leaf sample arising from control plants (**Figure 5A**), while it was slightly less or equal to 1 μm in diameter in leaf sample arising from salt grown plants (**Figure 5B**). Maximal stomatal opening of 5 μm diameter was found in leaf

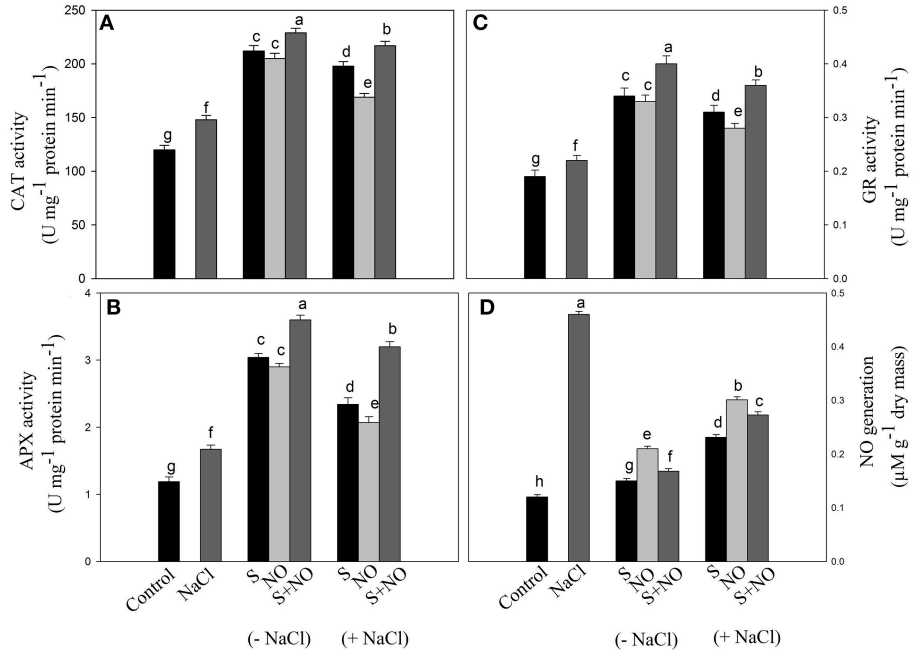

FIGURE 3 | Activity of CAT (A), APX (B), GR (C), and NO generation (D) in mustard (*Brassica juncea* L.) leaves treated with 100 μM nitric oxide (NO) and/or grown with S (200 mg S kg^{-1} soil; S) in presence or absence of 100 mM NaCl at 30 DAS. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$. APX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase.

TABLE 3 | Activity of ATPS, content of Cys, S and GSH, and redox state (GSH/GSSG) in mustard (*Brassica juncea* L.) leaves treated with 100 μM nitric oxide (NO) and/or grown with S (200 mg S kg^{-1} soil; S) in presence or absence of 100 mM NaCl at 30 DAS.

Treatments	ATPS (μmol g^{-1} protein s^{-1})	Cys (nmol g^{-1} leaf dry mass)	S (mg g^{-1} leaf dry mass)	GSH (nmol g^{-1} leaf dry mass)	Redox state (GSH/GSSG)
0	1.20 ± 0.064^{g}	5.9 ± 0.290^{g}	4.9 ± 0.232^{f}	57 ± 2.20^{g}	19.80 ± 0.612^{f}
NaCl	1.36 ± 0.043^{f}	6.8 ± 0.298^{f}	4.2 ± 0.119^{g}	69 ± 2.09^{f}	8.55 ± 0.643^{g}
S	2.27 ± 0.707^{c}	9.2 ± 0.312^{c}	7.5 ± 0.311^{c}	99 ± 2.57^{c}	32.00 ± 0.745^{c}
NO	2.20 ± 0.820^{c}	9.1 ± 0.308^{c}	7.3 ± 0.330^{c}	95 ± 2.45^{c}	31.30 ± 0.732^{c}
S+NO	3.60 ± 0.088^{a}	10.9 ± 0.442^{a}	8.8 ± 0.230^{a}	112 ± 2.80^{a}	39.20 ± 0.989^{a}
S + NaCl	1.94 ± 0.059^{d}	8.6 ± 0.307^{d}	6.1 ± 0.313^{d}	85 ± 2.33^{d}	24.90 ± 0.666^{d}
NO + NaCl	1.77 ± 0.065^{e}	7.6 ± 0.301^{e}	5.5 ± 0.300^{e}	78 ± 2.10^{e}	22.10 ± 0.654^{e}
S + NO + NaCl	3.10 ± 0.081^{b}	10.2 ± 0.370^{b}	8.4 ± 0.278^{b}	107 ± 2.73^{b}	37.30 ± 0.987^{b}

Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$. ATPS, ATP-sulfurylase; Cys, cysteine; GSH, reduced glutathione; GSSG, oxidized glutathione.

sample of plants grown with salt and treated with NO and S (**Figure 5C**).

The analysis of SEM showed that partially closure of stomatal aperture in salt grown plants was prevented by the application of NO together with S. Moreover, the frequency of occurrence of stomata was higher in plants treated with NO and S than control or salt treated leaves (**Figure 6**). Salt stress decreased stomatal frequency by 25.0% compared to control plants. The plants supplemented with NO and grown with S reversed the effects of salt stress and increased the stomatal frequency by 39.2% in comparison to control plants.

Ultrastructural Studies

Plants grown under normal condition were characterized with well developed thylakoid membrane system (**Figures 7A**,**D**). However, greater modifications were seen in the salt treated plants. Plants grown with salt showed distorted chloroplast thylakoid system (**Figures 7B**,**E**). The more significant differences in chloroplast ultrastructure were observed in plants treated with NO and S under salt stress. The distortions of chloroplast structure by salt were seen lesser when plants received NO plus S. The chloroplast structure was well developed with regular shape and well-arranged thylakoid systems. A large

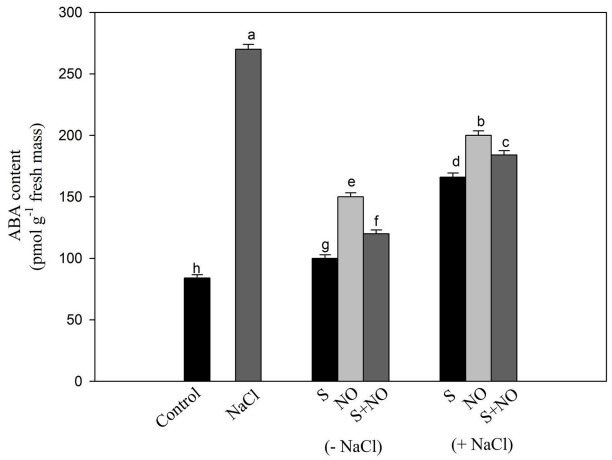

FIGURE 4 | Content of ABA in mustard (*Brassica juncea* L.) leaves treated with 100 μM nitric oxide (NO) and/or grown with S (200 mg S kg^{-1} soil; S) in presence or absence of 100 mM NaCl at 30 DAS. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

number of thylakoid stacks were observed in the chloroplasts compared to control and salt treated plants (**Figures 7C,F**).

Effect of NO and S on Growth Characteristics

Salt stress decreased leaf area and plant dry mass by 49.9 and 53.1%, respectively with salt stress compared to control plants, but this inhibition was found reversed in plants receiving NO or S. The addition of NO or S increased leaf area equally by 43.1% and plant dry mass by 37.7%, respectively compared to control plants. Under salt stress, the effects of S was greater increasing leaf area and plant dry mass by 32 and 27.7%, while NO could increase leaf area and plant dry mass by 16.3 and 14.8%, respectively compared to control plants. The maximum increase in leaf area and plant dry mass was obtained with the combined treatment of NO and S compared to control plants under no stress (**Figure 8**).

DISCUSSION

NO Reduces Ions Accumulation and Oxidative Stress in Plants Receiving S

Plants treated with NO plus S accumulated Na^+ and Cl^- in leaf and root to minimum under salt stress. The accumulation of ions in leaf was lesser than root showing that NO plus S treatment influenced the transport efficiency of ions. It has been shown that exogenous NO increased the activity of tonoplast H^+ ATPase and Na^+/H^+ antiport facilitating Na^+ compartmentation (Zhang Y. et al., 2006). The importance of NO in increasing the content of K^+, Ca^{2+}, and Mg^{2+} in salt-treated *Gossypium hirsutum* plants has been demonstrated (Dong et al., 2014a). It may be suggested that lowering of Na^+ in cytosol by NO plus S treatment was through regulation of expression and activity of Na^+ transporters and H^+ pumps that generated the driving force for the transport. Application of NO plus S decreased H_2O_2 content and lipid peroxidation maximally and enhanced salt tolerance in plants by enhancing activity of the antioxidant enzymes. Recently Fatma et al. (2014), reported that S supplementation lowered ions accumulation and enhanced GSH production resulting in removal of H_2O_2 (Fatma et al., 2014). Moreover, NO has also the potential to modulate antioxidant defense system for scavenging ROS under salt stress (Kopyra and Gwozdz, 2003; Fatma and Khan, 2014). NO together with S facilitated the membrane functions through the induction of the antioxidant system and increased GSH production. Supplementation of NO or S to salt

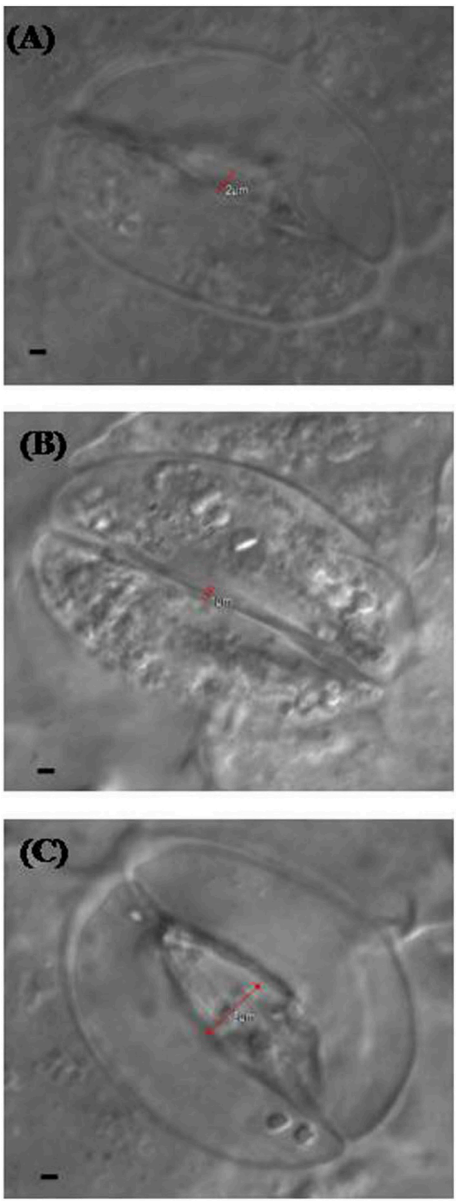

FIGURE 5 | Leaf stomatal response of mustard (*Brassica juncea* L.) under the effect of various treatments. The stomatal opening and closing response was studied using confocal microscopy in control **(A)**, 100 mM NaCl **(B)**, and S (200 mg S kg^{-1} soil; S) and 100 μM nitric oxide (NO) with 100 mM NaCl treated plants **(C)** at 30 DAS. Bar represents 1 μm in the panels **(A–C)**.

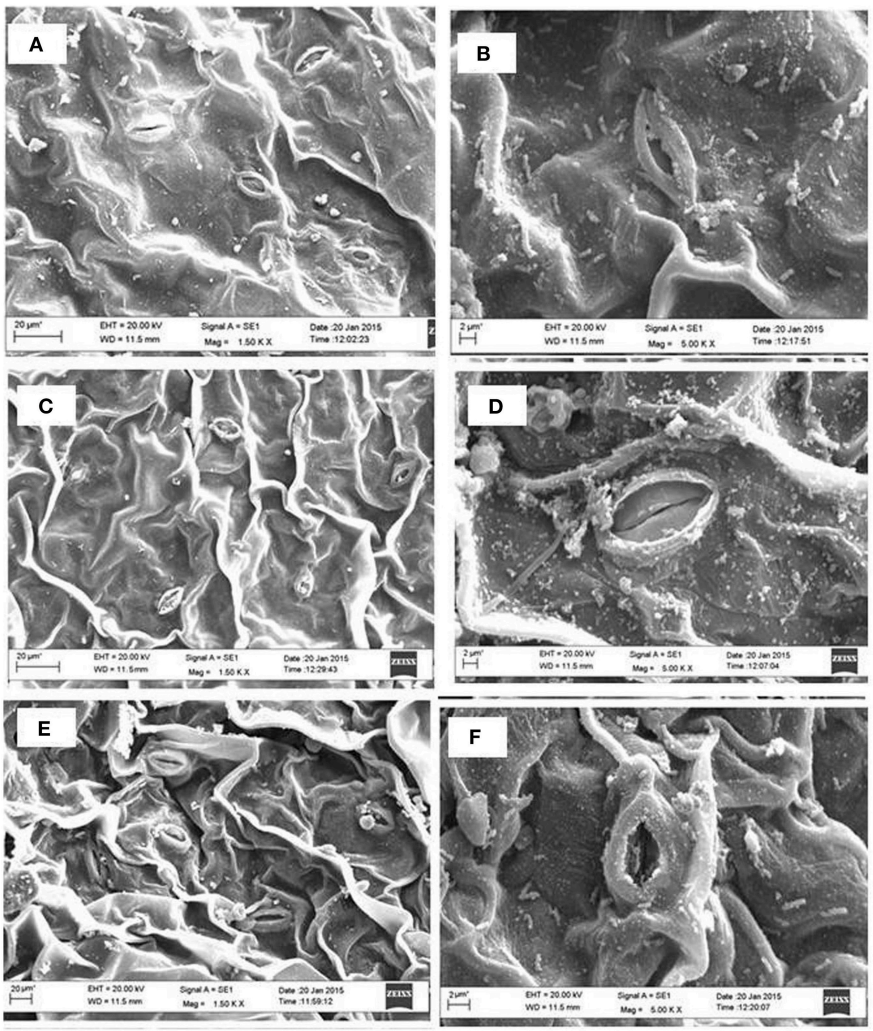

FIGURE 6 | Leaf stomatal behavior of mustard (*Brassica juncea* L.) performed under control (A,B), 100 mM NaCl (C,D), and S (200 mg S kg^{-1} soil; S) and 100 μM nitric oxide (NO) with 100 mM NaCl (E,F). The effect of stomatal opening and closing was observed under the scanning electron microscopes at 1.50 K X **(A,C,E)** and 5.0 K X magnifications **(B,D,F)** in the leaf surface of mustard (*Brassica juncea* L.) grown under 100 mM NaCl treated plants at 30 DAS.

grown plants promoted activity of CAT, APX, and GR which were important for the efficient scavenging of H_2O_2 and TBARS. Bai et al. (2011) have reported that NO reinforces APX and CAT activities and reduces Cd-induced carbonylation in *Antiaris toxicaria* seeds. The involvement of NO has been demonstrated in protection of *Triticum aestivum* roots against aluminum toxicity (Sun et al., 2014). Further, NO up-regulated the activity and transcription of APX and GR, the two key enzymes in the AsA-GSH cycle in *Nicotiana tabacum* (Zhang et al., 2009a) and *Cucumis sativus* leaves (Cui et al., 2011) and conferred resistance to abiotic stress.

NO Reduces Superoxide Accumulation

It was found that application NO and S reduced the production of ROS ($O_2^{\cdot-}$). The influence of NO and S on the inhibition of ROS assayed as superoxide accumulation has not been reported in the literature. The present study showed that application of NO and S on salt grown plants up-regulated the antioxidant system and prevented the accumulation of ROS. The role of NO has been recognized in detoxifying ROS either directly interacting with $O_2^{\cdot-}$ (Nakazawa et al., 1996) or enhancing function of the antioxidant system (Tewari et al., 2008). NO interacts with ROS such as $O_2^{\cdot-}$ and forms nitrating agent peroxynitrite, which serves as a signaling molecule in stress response and function in regulating protein activity (Baudouin, 2011). Bai et al. (2015) reported that NO-treated plants counteracted oxidative damage due to the decreased production rate of $O_2^{\cdot-}$ and reduced accumulation of H_2O_2.

NO develops Salt Tolerance by Enhancing S-assimilation

S-assimilation capacity of plants under salt stress was substantially increased with the combined application of NO and S resulting in increased activity of ATPS and content of

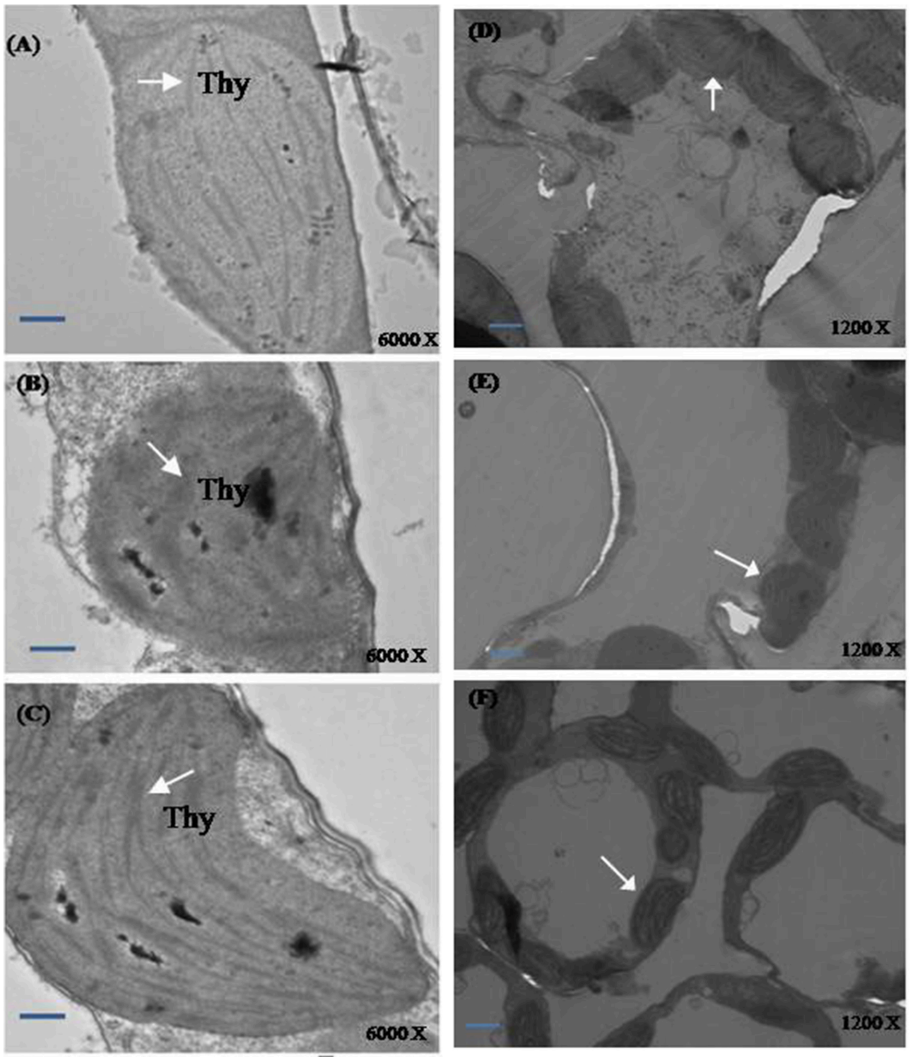

FIGURE 7 | Ultrastructure of chloroplasts from leaves of mustard (*Brassica juncea* L.). Transmission electron microscopy micrographs on the representative chloroplasts from the leaves of mustard performed on the control **(A,D)**; 100 mM NaCl **(B,E)** and S (200 mg S kg^{-1} soil; S) and 100 μM nitric oxide (NO) with 100 mM NaCl treated plants **(C,F)** at 30 DAS. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy operated at voltage of 120 kV and magnification of 6000 X and 1200 X. Bar represents 100 nm in the **(A–C)** and 500 nm in the **(D–F)**.(Thy; thylakoid membranes).

Cys and GSH and redox state. The studies on the involvement of NO in enhancing S-assimilation in plants supplemented with S under salt stress have not been reported earlier. In this study, NO influenced S-assimilation by regulating the NO generation in plants receiving S. It has been reported that NO stimulates γ-glutamylcysteine synthetase and GSH synthetase gene expression in S-assimilation and up-regulates the GSH production in *Medicago truncatula* (Innocenti et al., 2007) and S. *lycopersicum* (Wang et al., 2015b). Under stress condition, excess GSH readily reacts with NO to form GSNO, which serves as NO reservoir in plants (Chaki et al., 2009; Wang et al., 2015a). The formed GSNO is catalyzed by GSNOR producing GSSG and NH_3. The resulting GSSG is then reduced again to GSH by the NADPH-dependent reaction catalyzed by GR. Thus, a rapid recycling of GSH is related to the role of NO in maintaining the GSH pools as well as the ratio of GSH to GSSG. Hai-Hua et al. (2005) reported an increased GSH/GSSG ratio with exogenous application of NO in *T. aestivum* seedlings subjected to 150 mM NaCl stress. Exogenous NO increased GSH content in *S. lycopersicum* roots and leaves under copper (Cu) stress, adjusting the GSH/GSSG ratio (Wang et al., 2015b). Similarly, heat treatment resulted in decreased GSH/GSSG ratio which was reversed by the supplementation of NO (Hasanuzzaman et al., 2012). In *Oryza sativa* arsenic stress reduced GSH content and GSH/GSSG ratio and NO supplementation maintained the GSH/GSSG ratio (Singh et al., 2015). Additionally, the ability of exogenous NO to decrease lipid peroxidation has also been considered as one of the factors that maintains higher ATPS activity in *C. sativus* roots (Shi et al., 2007). Zhang et al. (2009b) showed that exogenous NO alleviated Cu toxicity by enhancing

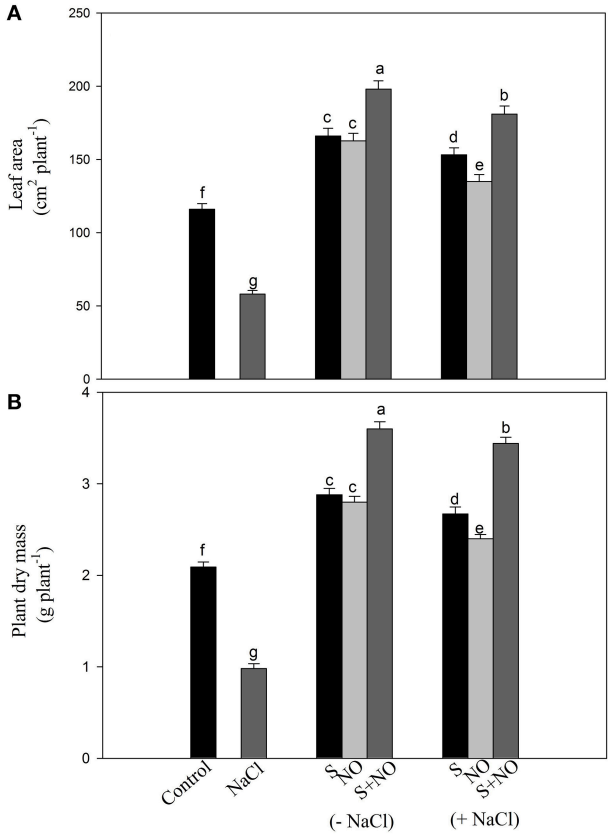

FIGURE 8 | Leaf area (A) and plant dry mass (B) of mustard (*Brassica juncea* L.) treated with 100 μM nitric oxide (NO) and/or grown with S (200 mg S kg^{-1} soil; S) in presence or absence of 100 mM NaCl at 30 DAS. Data are presented as treatments mean ± SE ($n = 4$). Data followed by same letter are not significantly different by LSD test at $P < 0.05$.

an antioxidative system and increasing ATPase activity in *S. lycopersicum*.

The Influence of NO and S on NO Generation

The maximal NO generation was found in plants grown with salt stress. Gould et al. (2003) have also shown that NO generation increased in *N. tabacum* plants in response to salinity stress. The increased NO accumulation with exogenous NO and responses of plants to abiotic and biotic stresses suggested NO as an important signal molecule (Delledonne et al., 1998; Zhou et al., 2005). The application of NO and S increased the synthesis of NO and stimulated the activity of antioxidant enzymes for the protection from salt induced-oxidative stress. Neill et al. (2008) suggested that various stresses such as drought and salinity induced NO generation which activated cellular processes for protection against oxidative stress.

NO and S Influence ABA Content, Stomatal Response, and Chloroplast Ultrastructure

Confocal and SEM analyses of plant samples revealed the potential role of NO and S in regulating the stomatal responses. Treatment of plants with salt induced stomatal closure and increased ABA accumulation, while application of NO and S together induced stomatal opening. These findings were also supported by the SEM images and also by much lesser increase in ABA in NO plus S treatment than salt treated plants. The stomatal frequency was also found higher with NO and S than both control and salt treated plants. In contrast to the present report that NO induced stomatal opening, Garcìa-Mata and Lamattina (2001) found stomata closed in *Vicia faba* under drought stress. The NO induced stomatal opening in the present study might depend on osmotic relationship in plants cells. The influx/efflux of Ca^{2+} ions controlled stomatal movement (McAinsh et al., 2000) and ABA concentration in guard cells (Wang, 2014). Sakihama et al. (2003) reported that NO was involved in the signal transduction mechanisms for opening of stomata in *V. faba*. ABA induced increase in activity of antioxidant enzymes has been found linked with NO generation showing the relationship between ABA and NO in oxidative stress tolerance in *Stylosanthes guianensis* and *Arabidopsis thaliana* (Zhou et al., 2005; Neill et al., 2008). The present study also confirms the relationship between ABA and NO under salt stress. However, NO induced ABA content was found dependent on S that controlled stomatal movement. The increased GSH content with NO was further improved with S that was involved in cellular redox homeostasis and stomatal movement. The relation between ABA and S-assimilation has been shown earlier (Jiang and Zhang, 2001), where the authors showed ABA mediated accumulation of GSH. Besides, NO itself increased the antioxidant activity to protect plant from the oxidative stress induced by salt stress (Li et al., 2008). It is likely that potential interrelation between ABA, NO, and S-assimilation controlled the stomatal movement.

A coordinated influence of NO and S on the ultrastructural changes of chloroplasts under salt stress has not earlier been described. The increase in size of thylakoid with misshaped structure was prevented by the application of NO and S under salt stress. Chloroplast structure showed a well-developed and regular shape with well-arranged thylakoid systems. The plants exhibited more chloroplasts per cell or more thylakoid membranes per chloroplast than the control or salt treated plants with NO plus S due to the presence of higher chlorophyll content and lower level of lipid peroxidation.

NO and S Improve Photosynthesis under Salt Stress

Plants receiving exogenous NO and grown with S showed improved photosynthetic performance in the absence of salt. However, maximal increase in photosynthesis was noted with the combined treatment of NO plus S under both no stress and salt stress conditions (**Table 1**). Under salt stress, NO influenced photosynthesis by regulating NO generation in plants receiving S. The individual or combined application of NO and S favored S-assimilation, GSH synthesis, optimal NO generation and redox state. Moreover, there was also influence on the stomatal movement. The NO increased GSH production provided an important regulatory loop for NO bioactivity leading to better

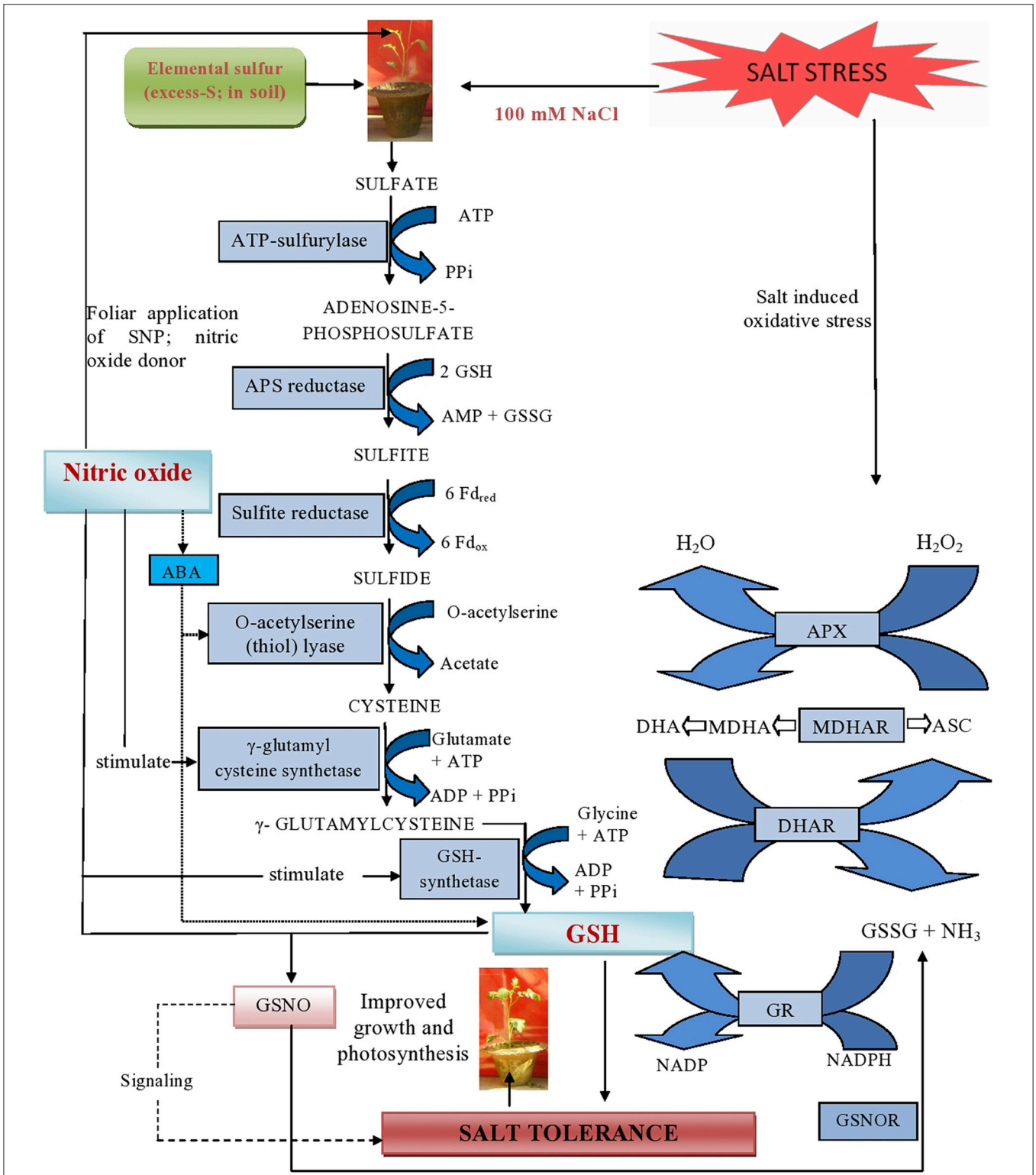

FIGURE 9 | Schematic representation of major mechanisms underlying excess-S and NO-mediated alleviation of salt stress in mustard (*Brassica juncea* L.). The figure shows that NO generated by the supplementation of SNP (NO donor) can react with GSH and yield S-nitrosoglutathione (GSNO). This metabolite can be converted by the enzyme GSNO reductase (GSNOR) into GSSG and NH_3. Additionally, supplementation of excess-S and NO reduced NaCl-induced oxidative stress and influenced photosynthesis by increasing GR-mediated conversion of GSSG into GSH and regulating NO generation in plants. Arrow (– – ▶) indicates signaling between NO and GSH for salt tolerance. Arrow (·····▶) indicates interaction between NO and ABA. NO induces ABA accumulation which in turn up regulates S-assimilation and controls GSH formation.

reduced cell environment under salt stress. Recently, it has been shown that application of NO increased photosynthesis through increase in stomatal conductance and Rubisco activity (Fatma and Khan, 2014). However, the increase in photosynthesis by exogenous NO and S under salt stress and the activity and SDS-PAGE profile of Rubisco and chlorophyll fluorescence are reported for the first time. Supplementation of NO and S was beneficial in increasing photosynthetic performance more than their individual effects because it maximally reduced the oxidative stress by increasing S-assimilation and antioxidants, and NO generation in suitable range. The involvement of NO in protection of chlorophyll against cadmium in *Helianthus annuus* (Laspina et al., 2005), drought stress in *O. sativa* (Farooq et al., 2009) and Cu in *Lolium perenne* (Dong et al., 2014b) has been reported. The application of NO has also resulted in improved gas exchange parameters and chlorophyll fluorescence under salt stress at seedling stage in *Lycopersicon esculentum* (Wu et al., 2011) and *H. vulgare* (Zhang L. et al., 2006). The response of NO has been linked with the availability of mineral nutrients. Wang et al. (2013) reported that NO increased uptake of Fe and magnesium was responsible for improving chlorophyll synthesis, photosynthesis and transpiration. Kong et al. (2014) reported that foliar application of NO and salicylic acid improved photosynthesis and supported Fe uptake, translocation and activation in *Arachis hypogaea*. Moreover, exogenous NO promoted the uptake and translocation of potassium, zinc and Fe mineral elements and stomatal aperture under Cu stress in *L. perenne* (Dong et al., 2014b). **Figure 9** explains how the application of NO reduced salt induced oxidative stress and promoted photosynthesis in plants receiving S.

The present study showed clear differences in Rubisco protein pattern in plants receiving NO and S in salt treated plants. Protein bands with higher intensity were obtained in plants treated with NO plus S under salt stress. Fatma et al. (2014) showed that S application resulted in assemblage of new proteins and increased intensity of protein band in *B. juncea.*

NO and S Improve Growth under Salt Stress

The inhibition of leaf area and plant dry mass was alleviated by the application of NO or S. Foliar spray of NO on various crops such as *Z. mays, O. sativa, L. esculentum* and *B. juncea* resulted in increased plant growth under salt stress that was due to high activity of antioxidant enzymes (Zhang et al., 2004; Farooq et al., 2009; Wu et al., 2011; Fatma and Khan, 2014). The study of Kausar et al. (2013) showed that the exogenous NO application proved beneficial in enhancing plant dry weight, length of both shoot and root of salt-stressed plants.

CONCLUSION AND FUTURE PROSPECTS

It may be concluded that NO or S improves photosynthetic performance of plants both under normal and salt stress conditions. Their combined application maximally alleviated salt stress induced effects on photosynthesis and growth of plants. The positive effect of the combined treatment of NO and S was through their influence on stomatal responses, S-assimilation and the antioxidant system. Thus, the utilization of S under salt stress was found as an essential key factor in activating the antioxidant system and GSH production. NO acted as signaling molecule which controlled the utilization of S and GSH production under salt stress. Therefore, their coordinated effect was more than their individual effect in alleviating salt stress and promoting photosynthetic and growth performance. Future strategies should be focussed to unravel the role of NO in regulating the various enzymatic steps of S-assimilation pathway and production of reduced S metabolites using molecular tools. It is also essential to examine the interaction between NO and other phytohormones in signaling stomatal response and regulation of photosynthesis. It is known that S-assimilation is linked to ethylene production through Cys synthesis and NO promotes S-assimilation. It is therefore, likely that NO and S regulated interaction between ABA and ethylene in guard cells that may increase the stomatal and photosynthetic response under salt stress although this remains to be tested.

AUTHOR CONTRIBUTIONS

MF designed and conducted the experiment, TP, AM helped in data analysis and presentation, while NK overall supervised the work and corrected the manuscript.

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Involvement of Inositol Biosynthesis and Nitric Oxide in the Mediation of UV-B Induced Oxidative Stress

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The involvement of NO-signaling in ultraviolet B (UV-B) induced oxidative stress (OS) in plants is an open question. Inositol biosynthesis contributes to numerous cellular functions, including the regulation of plants tolerance to stress. This work reveals the involvement of inositol-3-phosphate synthase 1 (IPS1), a key enzyme for biosynthesis of *myo*-inositol and its derivatives, in the response to NO-dependent OS in *Arabidopsis.* Homozygous mutants deficient for IPS1 (*atips1*) and wild-type plants were transformed with a reduction- *grx1-rogfp2* and used for the dynamic measurement of UV-B-induced and SNP (sodium nitroprusside)-mediated oxidative stresses by confocal microscopy. *atips1* mutants displayed greater tissue-specific resistance to the action of UV-B than the wild type. SNP can act both as an oxidant or repairer depending on the applied concentration, but mutant plants were more tolerant than the wild type to nitrosative effects of high concentration of SNP. Additionally, pretreatment with low concentrations of SNP (10, 100 μM) before UV-B irradiation resulted in a tissue-specific protective effect that was enhanced in *atips1*. We conclude that the interplay between nitric oxide and inositol signaling can be involved in the mediation of UV-B-initiated oxidative stress in the plant cell.

Keywords: oxidative stress, nitric oxide, inositol-3-phosphate synthase 1, sodium nitroprusside, reduction–oxidation-sensitive green fluorescent protein 2, glutathione; oxidized disulfide form of glutathione

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INTRODUCTION

Ultraviolet B (280–315 nm waveband of the solar irradiation) amounts to almost 2% of short-wave radiation reaching living organisms in most ecosystems, and this proportion is likely to increase due to the ozone layer depletion (Caldwell et al., 2003; McKenzie et al., 2011). UV-B overexposure leads to numerous harmful consequences in plant cells by damaging DNA (cyclobutane pyrimidine and pyrimidine (6-4) pyrimidinone dimers formation), the photosynthetic apparatus (thylakoid disruption and disintegration of the double membrane envelope surrounding the chloroplast, destruction of chlorophyll and carotenoids) and membranes that undergo lipid peroxidation.

Abbreviations: EZ, root elongation zone; GRX1, glutaredoxin; GSNO, *S*-nitrosoglutahtione; IPS1, inositol-3-phosphate synthase 1; NO, nitric oxide; OS, oxidative stress; roGFP2, reduction–oxidation-sensitive green fluorescent protein 2; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT, root tip area; SNP, sodium nitropusside.

Ultimately, these cellular damages can lead to programmed cell death (Lytvyn et al., 2010; Krasylenko et al., 2013), reduced growth and productivity and genomic instability (Kunz et al., 2006; Kataria et al., 2014). Interestingly, UV-B exposure can also improve plant resistance to pathogens (Paul, 2000; Kunz et al., 2006), probably due to the activation of defense mechanisms, which provides evidence for the activation of signaling cascades by UV-B exposure. Protective mechanisms induced by UV-B include the activation of secondary metabolism and more specifically the biosynthesis of phenylpropanoids and UV-B-protective carotenoids and flavonoids (Hollósy, 2002; Kovács and Keresztes, 2002; Frohnmeyer and Staiger, 2003).

Overproduction of ROS, namely, superoxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radicals, caused by UV-B exposure, have both damaging actions and signaling functions in plant cell (Gill and Tuteja, 2010; Hideg et al., 2013; Kataria et al., 2014). Indeed, accumulation of ROS leads to OS activates defense reactions such as synthesis of UV-absorbing phenolic compounds; activation of DNA repair mechanisms; activation of enzymatic and non-enzymatic ROS scavenging; and overexpression of UV-B sensitive oxidative defense genes (Hideg et al., 2013; Kataria et al., 2014). Despite the considerable role attributed to ROS in plant cells response to UV-B, other signaling mediators leading to the activation of cellular protective mechanisms are predicted to exist. Indeed, treatment of *Arabidopsis thaliana* plants with the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazolin-L-oxyl-3-oxide (PTIO) and/or with an inhibitor of NO synthase [N^G -monomethyl-L-arginine (L-NAME)] have been shown to prevent UV-B induced expression of the flavonoid biosynthesis enzyme chalcone synthase (*CHS*). This observation indicates that up-regulation of *CHS* by UV-B requires NO (Mackerness et al., 2001), even though the existence of NO synthase in plants is still an open question (Corpas et al., 2004). In the past decades, NO has been recognized as a messenger molecule in plants that is involved both in physiological processes and stress responses (for detailed reviews see; Wendehenne et al., 2004; Yamasaki, 2005; Arasimowicz and Floryszak-Wieczorek, 2007; Besson-Bard et al., 2008; Siddiqui et al., 2011). Notably, a number of reports provide evidence for the protective effects of NO in plants challenged with UV-B. These effects include UV-B mediated changes of NO synthase-like and nitrate reductase activity; protective action of exogenous NO donors; interrelation of NO and ROS signaling pathways; NO impact on the ROS scavenging system activity; and the role of NO in UV-B perception by plant cell (summarized in Yemets et al., 2015).

Moreover, NO signaling has been implicated in many fundamental cellular processes (photosynthesis, organelles motility, hypersensitive response and programmed cell death) and cross-talks have been identified between NO-signaling and numerous pathways such as cytosolic calcium signaling; cyclic adenosine diphosphate ribose and cyclic guanosine 5′-monophosphate; salicylic, jasmonic acids and ethylene; ROS signaling, namely hydrogen peroxide; and MAPK-, salicylic acid-induced protein kinases (Yemets et al., 2015). Promising but totally undiscovered is the interrelation between NO and metabolic/signaling pathways related to *myo*-inositol derivatives.

Myo-inositol derivatives play a critical role in eukaryotic cells as membrane structural lipids precursors and as signaling molecules. In plants these compounds are involved in a large number of cellular processes such as biogenesis of the cell wall and membrane structures, phosphate storage, cell signaling and cell resistance to external stressful factors (Loewus and Murthy, 2000). For example, *myo*-inositol participates to plant tolerance to salt and cold stress (Valluru and Van den Ende, 2011). Furthermore, *Arabidopsis atips1* mutants which are deficient for the 1L-*myo*-inositol-1-phosphate synthase (*AtIPS1*, E.C.5.5.1.4) and accumulate less than 10% of wild-type *myo*-inositol levels show light-dependent spontaneous cell death, and constitutive activation of biotic stress response genes (Meng et al., 2009; Bruggeman et al., 2014), providing further evidence for the role of *myo*-inositol or its derivatives in plant stress response. Some studies performed on animal models suggest that direct cross-talks may exist between UV-B and *myo*-inositol signaling. Indeed, inositol hexaphosphate was shown to inhibit UV-B induced activation of the transcription factors AP-1 and NF-κB, thereby affecting UV-B-dependent gene expression (Chen et al., 2001), moreover NO was shown to induce *myo*-inositol 1,4,5-trisphosphate synthesis in rat pancreatic cells (Tritsaris et al., 2000; Moustafa et al., 2011). In this work, we investigated the relationships between *myo*-inositol metabolism and NO signaling in the mediation of UV-B induced OS in *Arabidopsis thaliana,* by investigating the response of *atips1* mutants to nitrosative stress and UV-B exposure.

MATERIALS AND METHODS

Plant Material and Treatments

Arabidopsis thaliana Columbia 0 wild type (Col-0) and *atips1* mutant (Meng et al., 2009) were transformed with *rogfp2-grx1* (Meyer et al., 2007) by floral-dip as described by Clough and Bent (Clough and Bent, 1998). For OS and plant growth measurements, mutants deficient for *AtERCC1* (At3g05210, UV REPAIR DEFICIENT 7) (Dubest et al., 2004) were used as hypersensitive controls. Sterilized seeds were sown aseptically on half strength MS medium (Duchefa Biochemie, Netherlands) containing 10 g L^{-1} glucose and agar (0,8% w/v). Seedlings were grown in a climate chamber at 24°C under a 16h/8h day/night regime and a light intensity of 3200 lux. 7-day old seedlings were used in all experiments. Plants were treated with 10, 100 μM and 1mM SNP. Incubation and pretreatment with SNP before UV-B irradiation was performed under bright light during 1 h. Plants were irradiated onto solid MS medium by applying 34 and 81 kJ m^{-2} of UV-B as described in (Lytvyn et al., 2010). Impact of SNP and UV-B on seedling growth was measured using the ImageJ software.

CLSM Measurement of OS

Oxidative stress was measured by Confocal Laser Scanning Microscopy quantification of redox-dependent changes in the fluorescence of roGFP2. Briefly, this approach relies on a modified GFP with cysteine residues that alter its excitation spectrum depending on their oxidation status. Transition of these

residues from an oxidized to a reduced state induces a shift in the roGFP2 excitation peak from 405 to 488nm. Mentioned cysteine residues are substrates for GRX and this catalytic reaction mimics the equilibration of cellular glutathione redox buffer [reversible conversion of glutathione from the reduced form (GSH) to the oxidized form (GSSG) Meyer et al., 2007]. Ratiometric analysis of the roGFP2 excitation efficiency at 405 and 488 nm thus reflects the redox status of the cell. Plant samples were examined using a Zeiss LSM 510 META laser scanning confocal microscope equipped with lasers for 405, 488, and 543 nm excitation. When needed, additional staining with 1.5μg ml^{-1} propidium iodide (PI) was performed. Images were collected using a 20x lens (EC Plan-Neofluar 20x/0,5, Zeiss) in multi-track mode with line switching and averaging of two readings. Excitation of reduced roGFP2 and PI was performed in the same track equipped with 488- and 543 nm lasers, respectively. Oxidized roGFP2 was exited at 405 nm in the second track. Fluorescence for both oxidized and reduced roGFP2 was collected with a 505–530 band-pass filter. Power of lasers was adjusted to 20% for excitation of 488 nm and to 40% for 405 nm, respectively. In all experiments calibration procedures with reduction/oxidation of the probe using 10 mM H_2O_2 and 10 mM DTT were performed as described in Meyer and Brach (2009). Dynamic measurement of OS development was made under the same conditions with 60 0s intervals in each time series. Estimations of 405/488 nm ratios were performed using Carl Zeiss Laser Scanning Microscope LSM510 Release Version 4.0 SP2. Before calculations of pixel intensity in the area of interest, background level of fluorescence of both 405 and 488 lasers was adjusted to the same basal level. OS development was investigated in five tissues, namely, RT area, EZ, root vessels, and parenchymatous cells of hypocotyls and leaves. In the illustrations excitations of reduced and oxidized roGFP2 are represented with false colors: reduced protein was colored red and oxidized colored blue. All experiments were performed in five and more replications. The results were presented as mean ± SE; statistical analysis was done by Student *t*-test and *P* values less than 0.01 were considered as statistically significant.

DAB Staining

Staining with 3,3′-diaminobenzidine (DAB) was used as an additional approach to monitor ROS accumulation, notably H_2O_2. 0,2% DAB (Sigma, USA) solution filtered with 0,44 μm Millipore membrane was used. Plant samples were incubated for 1h in the staining solution and fixed in 70% ethanol. Samples were then placed in 50% chloral hydrate/glycerol solution (w/v) overnight and mounted for light microscopy.

RESULTS

Tolerance of *atips1* Plants to SNP-Induced OS

The main goal of this work was to identify possible cross-talks between *myo*-inositol metabolism and NO signaling. To tackle this issue, we first investigated the sensitivity of Col-0 and *atips1* plants to the NO donor Sodium Nitroprusside (SNP). To this end, we followed the redox status of the roGFP2 sensor (Meyer et al., 2007) in the wild type and mutant backgrounds. roGFP2 was derived from enhanced GFP (EGFP) by introducing two cysteines at positions S147 and Q204, which are located on β-strands and can form a disulfide bond when oxidized. This sensor thus displays redox-dependent changes in its excitation efficiency, the maximal excitation efficiency of the reduced and oxidized forms being 405and 488 nm, respectively (Meyer and Dick, 2010). Thus, the ratio of fluorescence intensity obtained upon illumination at 405 or 488 nm reflects the redox status of the cell cytoplasm, and more specifically of the glutathione pool. Cellular GSH pools are a major non-enzymatic antioxidant system, and relative abundance of the reduced (GSH) and oxidized (GSSG) forms is considered as a reliable indicator of OS level in the cell; in turn, roGFP2 equilibrates with the redox potential of the cellular glutathione buffer via GRX activity (Meyer et al., 2007). In our investigations roGFP2 fused with human Grx1; Grx1-roGFP2 was used to provide higher specificity and sensitivity to the reaction because roGFP reveals slow response to changes in redox potential itself (Gutscher et al., 2008; Meyer and Dick, 2010). In preliminary experiments, we observed that high concentrations of SNP (1 mM and above) induce oxidative/nitrosative stress in *Arabidopsis* (unpublished data), and the same effects were observed in yeasts and insect cells (Lushchak and Lushchak, 2008; Lozinsky et al., 2012). We thus chose to apply 1 mM SNP treatment to follow nitrosative/OS development in our lines. We followed changes in the redox status over time in above-ground and below-ground tissues and in various cell types.

In the wild-type, we observed differences in the basal cellular redox status depending on the tissues: cells of the RT, vessels, and aerial tissues being more oxidized than cells of the elongation zone (EZ). The basal redox status was similar in wild-type and *atips1* mutant plants in most cell types except in the vasculature and RT in which basal oxidation levels were slightly higher in the mutant (**Figures 1.1–1.4**). Upon SNP treatment, we did not observe any OS development in leaves or hypocotyls (**Figure 1.4**). By contrast, root cells of the RT and EZ showed clear OS development in the wild-type, but not in the mutant where the redox status remained stable (**Figures 1.1–1.3**). In vascular tissues, OS levels remained unchanged in *atips1* but increased in the wild-type until they reached higher levels than in the mutant (**Figure 1.3**). Overall, the *atips1* was thus found to be tolerant to the nitrosative effects of SNP in all root cell types.

Redox Effects of SNP are Highly Tissue Specific and Are Altered in the *atips1* Mutant

To determine the sensitivity of various cell types to different amounts of NO-donor, an investigation of OS level was performed after 24 h of SNP treatment. Analysis was performed at late time points because the cellular redox status at the earlier time points was highly heterogeneous (not shown). This observation can be explained by the variability of the initial starting oxidation level in different plants that was leveled within 24 h treatment. SNP had either oxidizing or reducing action depending on the applied concentration (**Figure 2**). Ten and 100 μM SNP did not significantly modify the redox status of

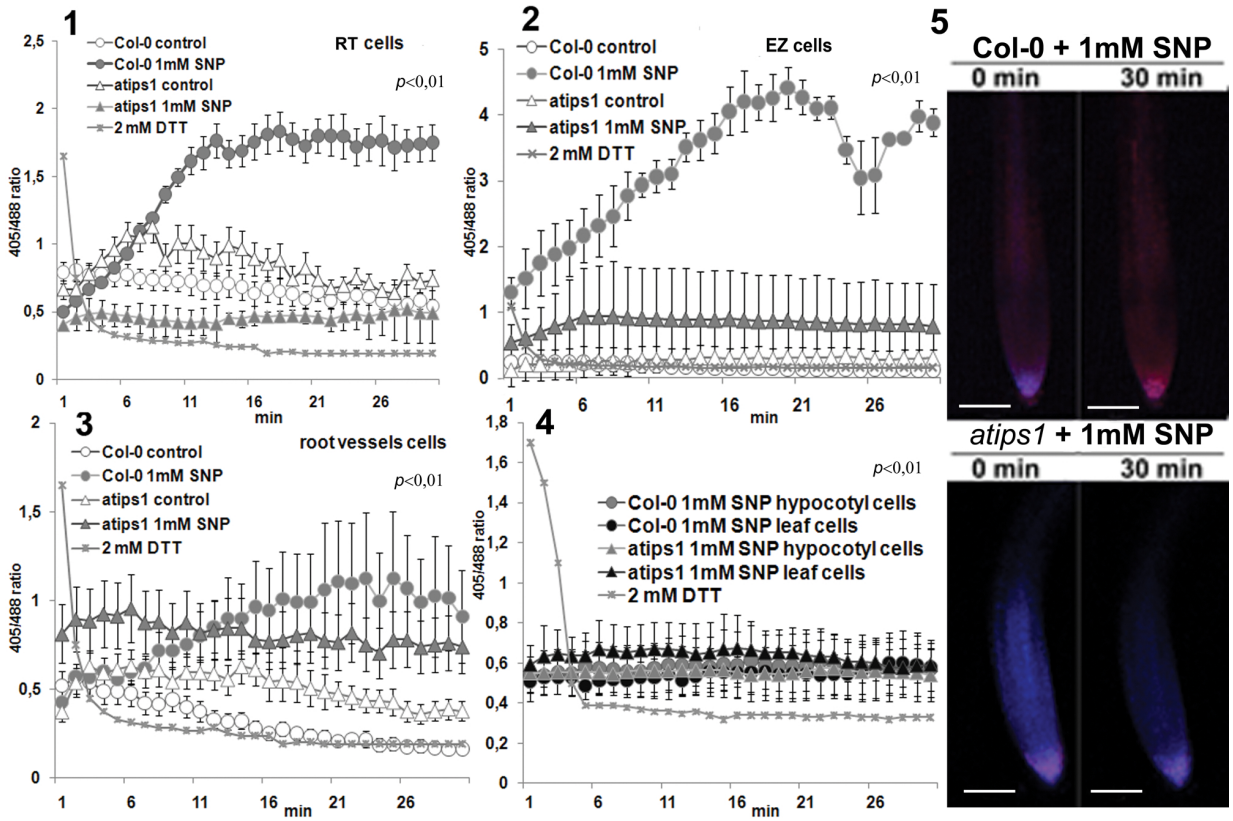

FIGURE 1 | Dynamics of roGFP2 oxidation in RT cells (1), root EZ cells (2), root vessels (3), hypocotyl/leaf cells of Col-0 (4) and *atips1* under 30 min treatment with 1 mM SNP (5). (5) Confocal overlay of the roGFP2 emission under 1 mM SNP treatment in the first and last time points. Notice almost intact redox status in the mutant, swift OS development in wild type since the initial treatment point and high initial oxidation level in RT cells. "2 mM DTT" in the legend displays calibration control of the method: sample oxidized with 10 mM H_2O_2 and subjected to 2 mM DTT treatment just before mesurment. Bar – 100 μM.

roGFP in the wild type, but induced significant reduction in root cells of the *atips1* mutants both in the RT and in the EZ cells. Other tissues were characterized by weak differences with a tendency to reduction in treated cells. After treatment with 1 mM SNP, both Col-0 and *atips1* plants exhibited near similar OS development in roots, but statistically significant OS development was also detected in mutant plants under the same conditions in hypocotyls and leaves.

Together, obtained data show that SNP has highly dose-dependent effects, low doses leading to a reduction of the cytoplasm whereas high doses induce OS. Furthermore, our results suggest that a signaling and/or metabolic link between *myo*-inositols and NO-induced OS exists in plant cells, and that this interrelation is strongly tissue-specific.

Prevention of UV-B Induced Oxidation by Pretreatment with SNP is More Effective in *atips1*

Because NO signaling has been involved in the cellular response to UV-B, we next investigated the interplay between *myo*-inositol accumulation and NO in this process. Determination of OS levels immediately after plants irradiation with 34 and 81 kJ m^{-2} of UV-B did not reveal statistically relevant changes compared to untreated plants. Therefore, UV-B oxidation effects were probed after 24h of treatment. Obtained results are summarized on **Figure 3**.

In the wild-type, UV-B irradiation induced OS in all analyzed tissues, root tissues, and particularly the RT and EZ being the most sensitive, and vascular cells the most tolerant. Hypocotyl cells showed some UV-B dose-dependent changes of oxidation levels that were more pronounced in the wild type, whereas in mesophyll cells, UV-B irradiation did not cause statistically significant OS. At 34 kJ.m^{-2}, *atips1* mutants displayed greater resistance to the harmful action of UV-B. Indeed in the wild type, UV-B irradiation caused a 2,5 fold and 4,9 fold increase of OS level in RT cells and cells of the EZ, respectively, whereas this increase was absent in the RT and only about twofold in the EZ in the *atips1* mutant. These results suggest that *atips1* is tolerant to the oxidative effect of UV-B.

Pretreatment of wild-type plants with SNP revealed clear anti-oxidizing effects under UV-B irradiation (**Figure 3A**). Indeed, incubation of Col-0 plants with both 10 and 100 μM SNP before irradiation at 34 kJ m^{-2} resulted in a sharp reduction of OS development: OS could no longer be observed in any of the tested cell types. The reducing action of SNP was so effective that

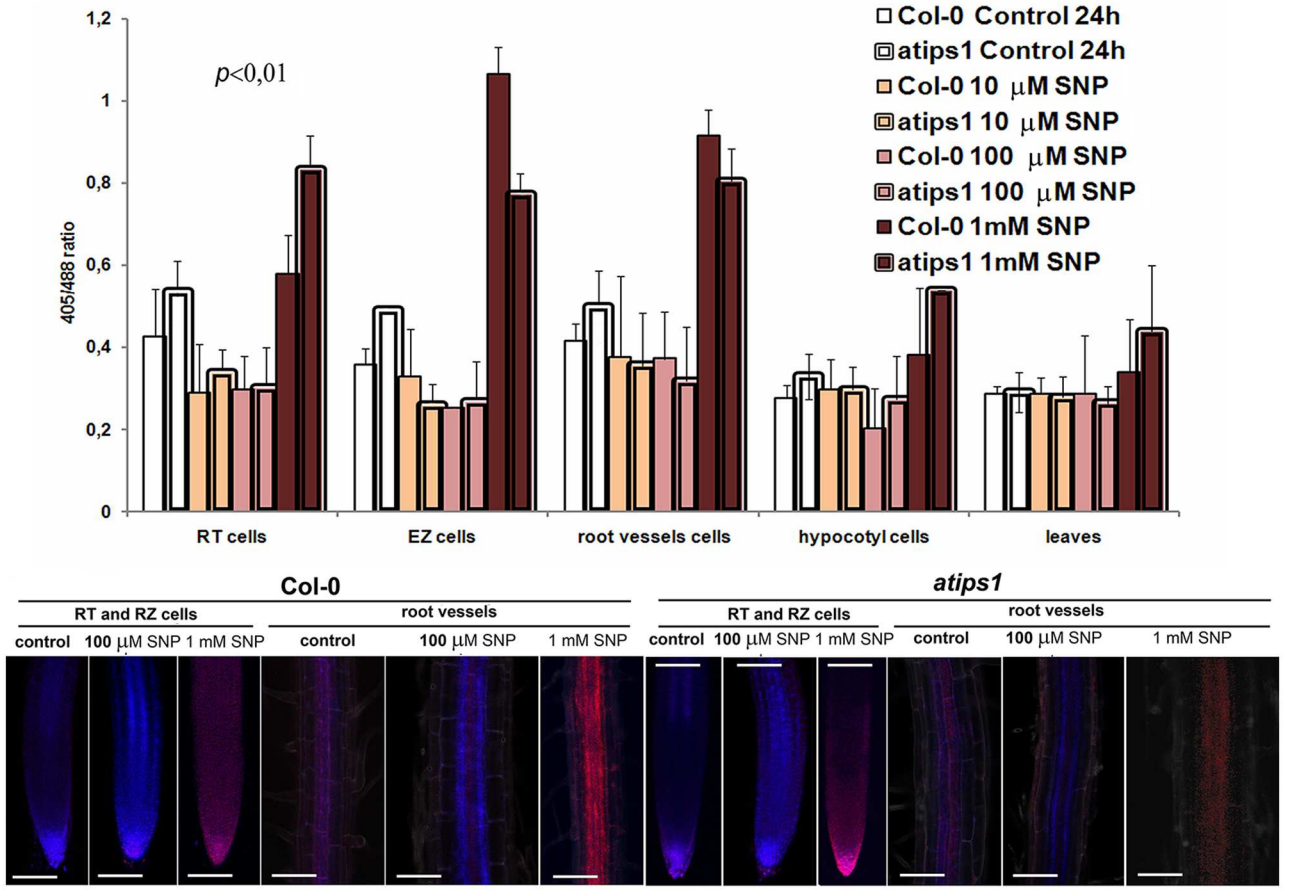

FIGURE 2 | Oxidizing and reducing tissue-specific effects of 24 h treatment of Col-0 and *atips1* seedlings with different SNP concentrations. Lower panel displays an example of confocal overlay of roGFP2 oxidation/reduction in root tissues. Bar – 100 μM.

oxidation levels in pretreated irradiated samples were even lower than in unirradiated plants. A similar response was observed in *atips1* plants (**Figure 3B**), but the anti-oxidant effect of SNP treatment was less pronounced in *atips1* due to the higher basal tolerance of the mutant to UV-B. Pretreatment of above-ground tissues led to similar reducing patterns, but had low intensity (**Figure 3A**).

Generally, pretreatment with SNP had no reducing effects in most tissues of wild type plants irradiated at 81 kJ.m^{-2}. By contrast, a statistically significant protective impact of NO was observed in the EZ and root vessels cells of *atips1* pretreated with 100 μM SNP. Indeed, roGFP2 in the cells of EZ was in 1,9 times more reduced after irradiation than in untreated plants. Cells of *atips1* vessels showed increased OS in response to this dose of UV-B compared to the wild type, due to the lower basal reduction of the reporter. In this cell type, pre-treatment with SNP again resulted in a protective effect against the oxidative effect of NO (**Figure 3B**). Together, results obtained after irradiation with higher UV-B dose support the notion that *myo*-inositol metabolism affects UV-B-induced OS.

Quantitative features of oxidative UV-B impact and protective effects of SNP were confirmed by qualitative staining of hydrogen peroxide as one of the main messenger ROS molecules (Van Breusegem et al., 2008). In these experiments, the ultraviolet sensitive *AtERCC1* mutant was used as an additional control. In this mutant, hydrogen peroxide accumulation was very low and showed little variation under all combinations of treatments (**Figure 4C**). Pretreatment of both Col-0 and *atips1* seedlings with 100 μM SNP lead to a decrease in H_2O_2 accumulation induced by 34 kJ m^{-2} of UV-B in root tissues, but only in mutant plants was this effect still observed after irradiation with 81 kJ m^{-2}.

To investigate the physiological relevance of the above-described observations, we monitored the effect of combined UV-B and SNP treatment on organ growth. Three-day-old Col-0, *atips1*, and *atercc1* seedlings subjected to treatment with different concentration of SNP, UV-B irradiation and combined SNP pretreatment and UV-B were grown for four days for time-course analysis of roots and hypocotyls growth. Control *atercc1* plants demonstrated the highest sensitivity to SNP: all inhibited both root and hypocotyls growth (**Figure 5.3**). In addition Col-0 and *atips1* seedlings treatment with 100 μM SNP also led to a small but statistically significant suppression of root growth (**Figures 5.1,2**). We therefore used 10 μM SNP as working concentration for combination with UV-B irradiation in growth

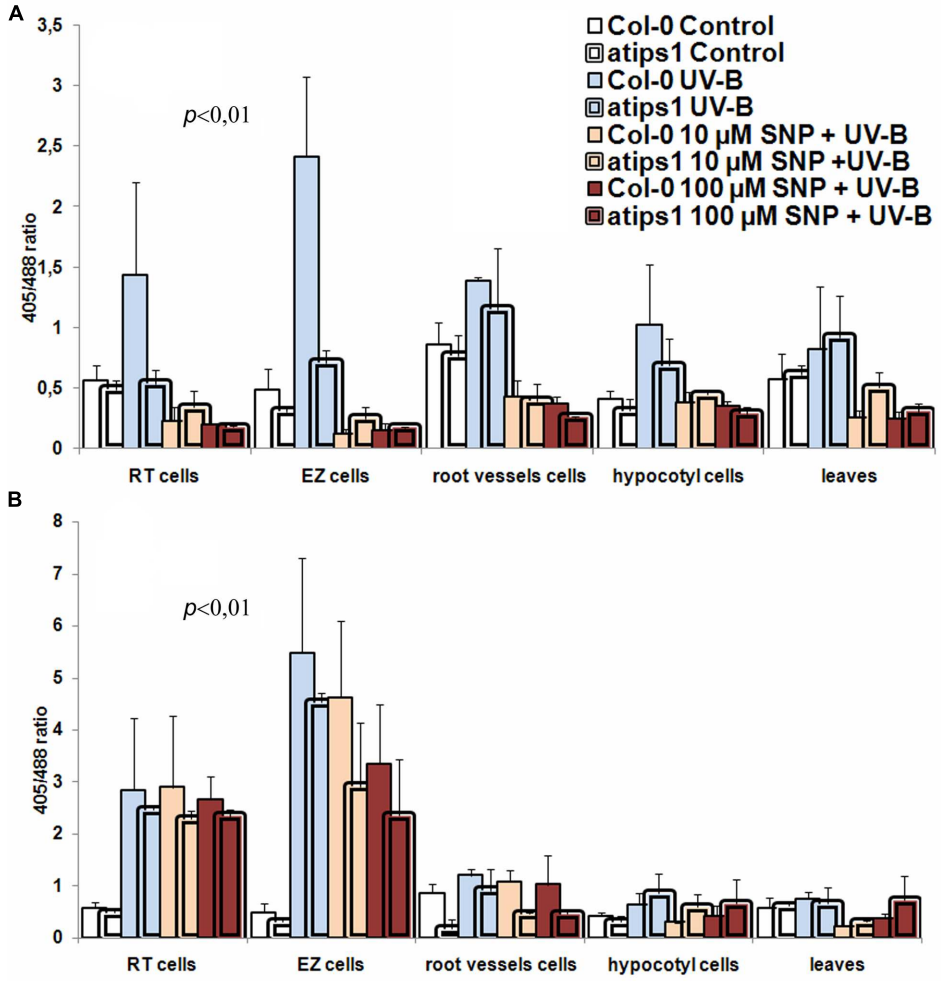

FIGURE 3 | Oxidative stress development and protective effects of SNP in plants irradiated with 34 kJ m^{-2} (A) and 81 kJ m^{-2} (B) of UV-B.

experiments. Inhibitory impact of irradiation and protective effect of SNP were observed upon plant treatment with 34 kJ m^{-2} of UV-B, but it was not pronounced or statistically valid, probably because of the low cytotoxity of this UV-B dose. 81 kJ m^{-2} of UV-B led to an almost complete arrest of seedlings growth in all lines. However, pretreatment with 10 μM SNP resulted in partial recovery of seedlings growth (roots of Col-0, and roots and hypocotyls of *atips1*) and this effect was also more pronounced in *atips1* plants (**Figure 5.4**).

DISCUSSION

To summarize, our results allowed us to define several aspects of plant response to SNP and UV exposure. We demonstrate that NO has highly tissue-specific effects, and that its cellular impact is modified according to *myo*-inositol accumulation. In the wild-type, RT and EZ cells were extremely sensitive to oxidation caused by high SNP concentrations, and this effect was completely abolished by *myo*-inositol deficiency; vascular cells were less sensitive to SNP but *atips1* mutants were also more tolerant than the wild-type; after 24 h of treatment similar oxidation patterns were observed in root tissues of both wild type and mutant plants, but mutants also showed OS development in underground tissues (**Figures 1** and **3**); SNP in low (10 and 100 μM) concentrations had a protective effect toward UV-B (**Figures 3** and **4**); *atips1* mutant was both more tolerant to the oxidative impact of UV-B and more perceptive to the protective action of NO under UV-B irradiation (**Figures 3–5**). Altogether these findings strongly suggest direct influence of *myo*-inositol metabolism on the NO-mediated stress signaling.

The validity of these results is supported by the use of a redox-sensitive GFP probe that is an extremely sensitive tool allowing accurate *in vivo* analysis of redox fluctuations over time. Because the roGFP2 sensor used in this study was fused to Grx1, its redox status reflects the redox status of the cellular glutathione pool (Gutscher et al., 2008; Meyer and Dick, 2010). Using the roGFP2 probe is appropriate in the investigations of NO impact on the cellular redox status because of the tight interrelation between GSH homeostasis and NO levels. Indeed,

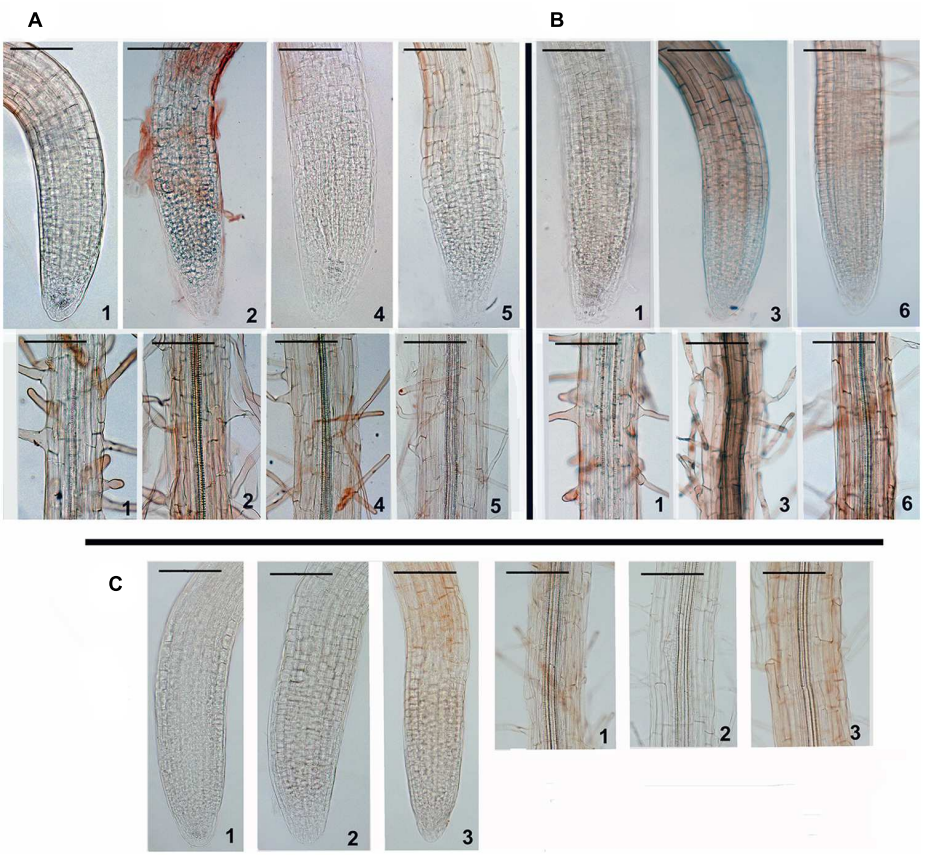

FIGURE 4 | Synergistic impact of SNP and UV-B on the intracellular level of hydrogen peroxide in Col-0 (A), *atips1* (B), and *atercc1* (C) plants (DAB staining): 1-control; 2- irradiation with 34 kJ m^{-2} UV-B; 3- irradiation with 81 kJ m^{-2} UV-B; 4- pretreatment with 10 μM SNP before irradiation with 34 kJ m^{-2} UV-B; 5- pretreatment with 100 μM SNP before irradiation with 34 kJ m^{-2} UV-B; 6- pretreatment with 100 μM SNP before irradiation with 81 kJ m^{-2} UV-B. Brown color intensity reflects H_2O_2 level in the cell.

in neuronal cells, GSH decrease was shown to cause protein nitration, *S*-nitrosylation, DNA breaks (Aquilano et al., 2011a) and to induce cell death in response to neurotrophic doses of NO (Canals et al., 2001, 2003). These data support the idea that GSH is the most important buffer of NO toxicity in neuronal cells, and cellular redox buffering controlled by GSH makes neuronal cells susceptible to endogenous physiological flux of NO (Aquilano et al., 2011a,b). In addition, close crosstalk between stressful oxidative and nitrosative signaling is generally accepted. Multiple lines of evidence confirm that ROS and RNS, namely, eroxyinitrite ($ONOO^-$), nitrogen dioxide ($.NO_2$), dinitrogen trioxide (N_2O_3) and *S*-nitrosoglutathione) in plants act together to modulate cellular responses to environmental stimuli. First RNS and ROS pools are linked both by direct chemical interaction between ROS and RNS and by interlinking molecules such as polyamines. Notably, GSNO is recognized as a key element in the interplay between the ROS and RNS metabolisms: GSNO is considered as an intracellular NO depot which is formed by NO's *S*-nitrosylation reaction with reduced glutathione (GSH; Corpas and Barroso, 2013). Second, there is significant overlap between the ROS/RNS-responsive gene networks and ROS/RNS-responsive proteins. Finally, ROS- and RNS-based stressful post-translational modifications of the proteins also have similarities (Molassiotis and Fotopoulos, 2011; Corpas and Barroso, 2013).

Our data highlighting oxidative impact of NO excess is consistent with the previously reported harmful effects of high NO levels in plant cells. NO has been shown to induce membrane damaging, DNA fragmentation, and reduction of photosynthesis and respiration; moreover, NO concentrations higher than 10 μM were shown to impair leaf expansion as well as shoot and root growth, and to induce changes in thylakoid viscosity, to impair photosynthetic electron transport, and to cause DNA damage and cell death (summarized in (Siddiqui et al., 2011)). Indeed, 1 mM SNP was an extremely cytotoxic amount and this treatment led to both clear OS development and total inhibition of root and hypocotyls growth in all examined *Arabidopsis* lines. However, under lower concentration (10 and 100 μM SNP) treatments two distinct (sort- and long-term) plant responses were observed. Short term (30 min) incubation with mentioned concentrations had no reliable effects on redox levels (not shown), and even induced mild reduction in root tissues (especially in *atips1*) within 24 h of treatment (**Figure 2**); but after four days

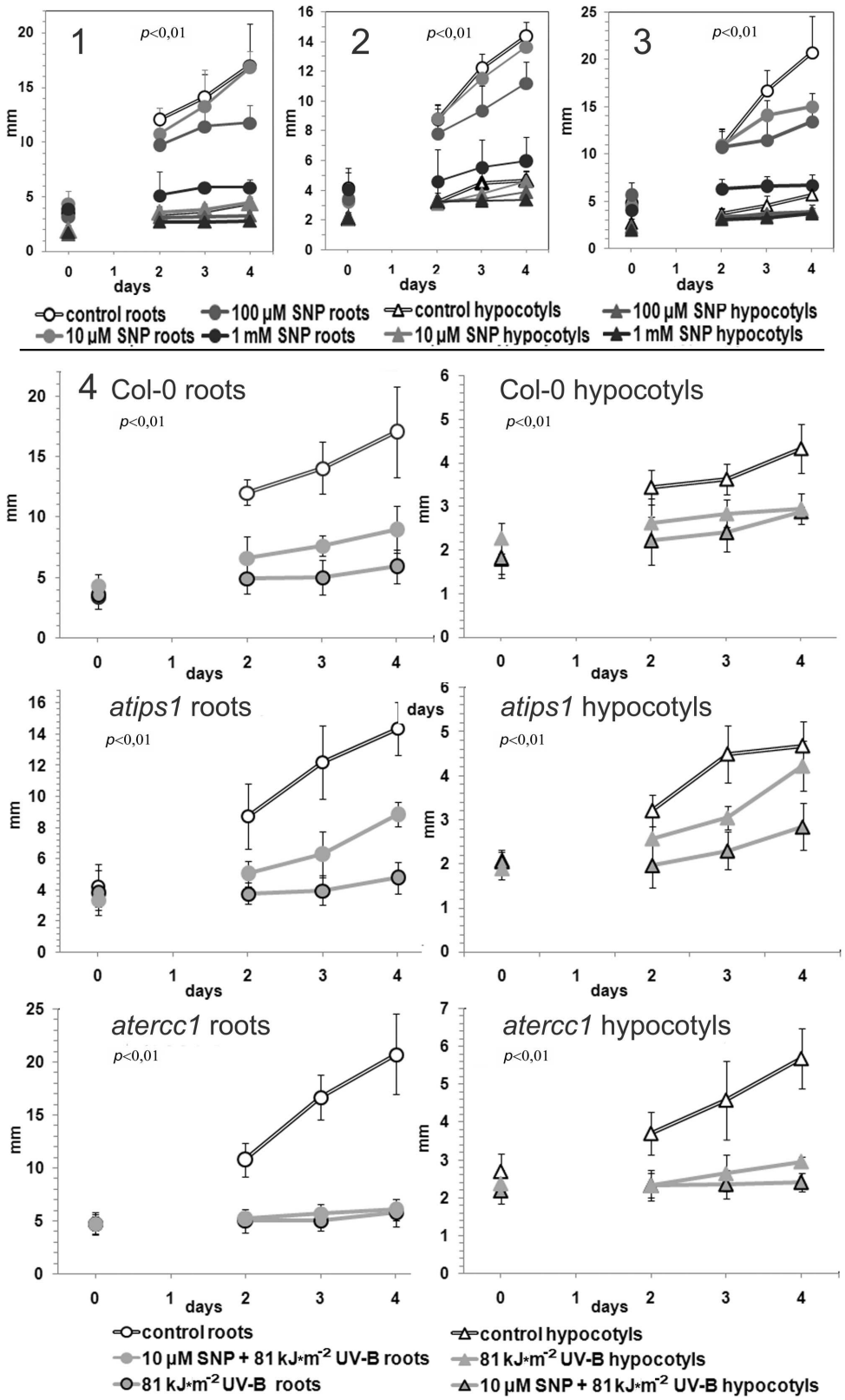

FIGURE 5 | Growth rates of wild type plants (1), and *atips1* (2), *atercc1* (3) mutants treated with different concentrations of SNP; and all mentioned lines that were irradiated with 81 kJ m^{-2} UV-B alone and with 10 μM SNP pretreatment (4).

following treatment statistically valid inhibition of root growth was observed in all lines (**Figures 5.1–5.3**). Notably, in *atercc1* the same effect was observed even after treatment with 10 μM SNP (**Figure 5.3**). ERCC1, is involved in the removal of non-homologous tails in homologous recombination (Dubest et al., 2002, 2004) and recombination−related DNA repair (Hefner et al., 2003), it is thus logical to suggest mentioned processes as one of the downstream targets of NO signaling and/or RNS impact.

One of the main findings discovered in this work is that the protective NO action against UV-B irradiation varies according to cell type. Indeed in our experiments, UV-B overexposure led to dose-dependent OS development that showed clear tissue specificity: RT cells were more sensitive to UV-B treatment than EZ cells or leaf parenchyma for example, consistent with the activation of anti-oxidant system in photosynthetic tissues. Interestingly, *atips1* was more resistant to NO induced OS than the wildtype, in line with the previous observation that this mutant is tolerant to OS induced by paraquat (Meng et al., 2009). OS generated by UV-B exposure also caused increased H_2O_2 production (**Figures 4A2, B3**), and plant growth inhibition. However, plant pretreatment with 100 μM SNP led to leveling and even decreasing to lower then initial redox state in all examined tissues of Col-0 and *atips1* under 34 kJ m^{-2} irradiation (**Figure 3A**). NO pre-treatment could not prevent UV-B induced OS in wild-type plant, but was able to reduce roGFP2 oxidation in the *atips1* background exposed to extreme (81 kJ m^{-2}) doses of UV-B (**Figure 3B**). Again, this finding provides evidence for the greater tolerance of the *atips1* mutant to OS: in this background, NO pre-treatment likely activated pathways involved in the protection against OS, thereby resulting in lower OS after UV-B treatment. Moreover, analysis of plant growth after UV-B exposure confirmed these hypotheses: the protective effect of NO pretreatment was more pronounced in *atips1* where in contrast to Col-0 it was observed both in roots in hypocotyls (**Figure 5.4**). Whether contrasting *myo*-inositol levels can account for the tissue specificity of NO response remains an open question. Indeed, *myo*-inositol quantification has been performed in leaves of the *atips1* mutant and has revealed a drastic reduction of the *myo*-inositol content. *Myo*-inositol accumulation is likely to be also severely affected in roots since the atips1 mutant has a root growth phenotype that can be rescued by addition of *myo*-inositol to the growth medium (Meng et al., 2009). These findings support the view that the higher tolerance of *atips1* to NO is indeed attributable to the reduction *myo*-inositol accumulation, but available methods for *myo*-inositol quantification do not allow to correlate the response of individual cell types to their *myo*-inositol levels.

A number of reports shed light on the underlying mechanisms that may account for the protective role of NO toward UV-B cytotoxicity. In plants, NO is involved in the tolerance to salinity, drought, ultraviolet, temperature, and heavy metals stresses (Siddiqui et al., 2011). Abiotic stresses provoke changes in NO levels directly or via ROS and hormonal mediation that, in turn, leads to activation of antioxidant enzymes expression and elimination of superoxide anions and lipid radicals (Siddiqui et al., 2011). Nitric oxide synthase (NOS) activity was shown to be significantly increased by UV-B irradiation, suggesting that NO may act as a secondary messenger under UV-B irradiation (Yemets et al., 2015). In plants, action of exogenous NO (namely, using SNP as a donor) under UV-B irradiation was shown to allow photosystem II protection and ROS scavenging (Shi et al., 2005; Wang et al., 2008). Described scavenging mechanisms include regulation of GSH accumulation, elimination of superoxide anions, increase of superoxide dismutase, catalase and peroxidase (Xue et al., 2007) as well ascorbate peroxidase (Santa-Cruz et al., 2014) activities; up-regulation of antioxidant heme oxygenase (Santa-Cruz et al., 2010) and decrease of nitrogenase activity (Xue et al., 2011). Recently, complex analysis of synergistic impact of SNP and UV-B in lettuce seedlings revealed that the above-mentioned pretreatment resulted in enhanced antioxidant enzyme activities, total phenolic concentrations, antioxidant capacity and expression of phenylalanine ammonia lyase; alleviation of chlorophylls, carotenoid, gibberellic and indole-3-acetic acid content inhibition, as well as in decrease of abscisic and salicylic acids (SA), malondialdehyde, hydrogen peroxide and superoxide anion (Esringu et al., 2015). In addition, earlier we have shown that exogenous NO protects organization of plant microtubules against disrupting effects of UV-B (Krasylenko et al., 2012).

NO-dependent changes in H_2O_2 accumulation were another notable finding of this work. As a rule, root tissues of intact plants were characterized by near undetectable H_2O_2 which was dramatically increased following irradiation with both UV-B doses (aboveground tissues were not investigated because of high basal levels of H_2O_2) (**Figures 4A1,B1**). SNP pretreatment of wild type and mutant plants before exposure to 34 kJ m^{-2} of UV-B completely abolished H_2O_2 production (**Figures 4A4,A5**). But only pretreated mutant plants demonstrated a clear decrease of H_2O_2 production, when irradiated with a higher UV-B dose (**Figures 4B3,B6**). This result is consistent with the above-mentioned role of exogenous NO in the regulation of catalase and peroxidase activity, and the finding that inhibition of NOS activity leads to similar consequences under UV-B irradiation (Kim et al., 2010). It is worth noting that in *atercc1* plants the effects of UV-B irradiation were totally different from Col-0 and *atips1*. Indeed, UV-B impact had no effects on H_2O_2 accumulation *in atercc1* compared to control (**Figures 4C1–4C3**). Since this line is hypersensitive to UV-B (**Figure 5.4**), we can assume that H_2O_2 may also play a signaling role in the UV-B response. Indeed, NO and H_2O_2 were identified as important early upstream signaling components which regulate expression of different sets of genes involved in defense and tolerance to UV-B radiation (Mackerness et al., 2001). The production of both molecule is known to be activated by various stresses and their synergistic signaling activity is cross-regulated with abscisic, jasmonic, salicylic acid and ethylene (Neill et al., 2002). H_2O_2 was proposed as a signal molecule in the mediation of excess excitation energy stress in *Arabidopsis* and this mediation is linked with changes in the redox state of the cellular glutathione pool (Mullineaux

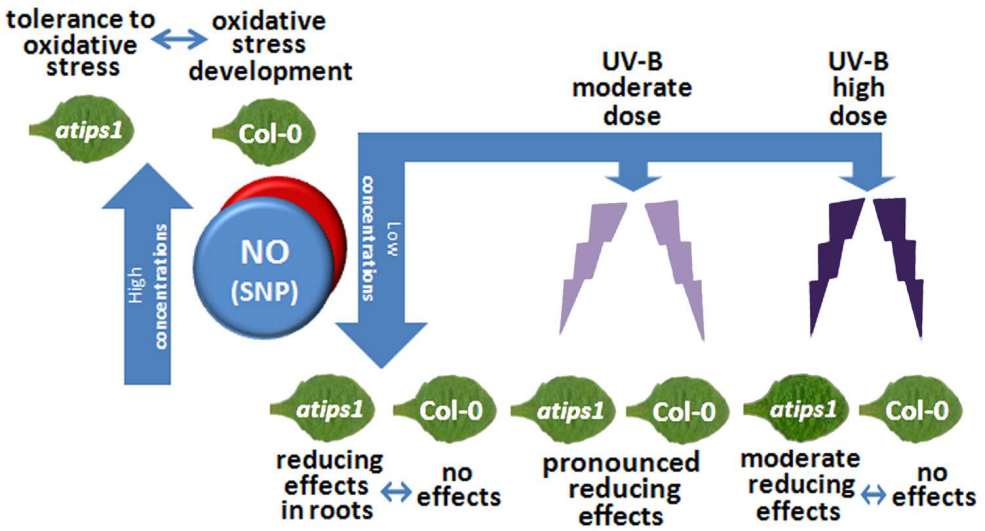

FIGURE 6 | Summarize dose-dependent redox behavior of Col-0 and *atips1* plants under SNP treatment and UV-B irradiation. IPS1-difficient plants demonstrate both tissue-specific tolerance to NO excess and higher receptivity to protective NO effect under ultraviolet irradiation.

et al., 2000). However, the above-described phenomenon of more pronounced NO influence effects on H_2O_2 homeostasis in *atips1* denotes the unknown interplay between *myo*-inositol and NO signaling pathways in stress-related regulation of H_2O_2 level.

The most debatable finding of this work is the unexpected resistance of *atips1* plants to NO and UV-B induced OS as well as its enhanced receptivity to protective effects of exogenous NO. Interestingly, resistance of the mutant is stimuli-specific: *atips1* mutants are tolerant to paraquat (Meng et al., 2009), but previously, using the same roGFP2-GRX expressing lines, we observed that *atips1* plants have considerably higher sensitivity to OS induced by SA (Lytvyn et al., 2011). These contrasting behaviors probably relate to the conditional cell death phenotype observed in *atips1*: exposure to long days or high light intensity induces a peak in SA production, leading to lesion formation in the mutant (Meng et al., 2009; Donahue et al., 2010). Thus this mutant shows enhanced basal tolerance to OS, but SA accumulation elevates OS above a threshold leading to cell death.

In animal cells, the protective role of *myo*-inositols toward exposure to UV-B is well documented. Indeed, inositol hexaphosphate (InsP6) prevents activation of activator protein-1 (AP-1) and NF-kappaB as well as phosphorylation of extracellular signal-regulated protein kinases (Erks) and c-Jun NH2-terminal kinases (JNKs) in response to UV-B, thereby avoiding UV-B-induced carcinogenesis (Chen et al., 2001). Additionally, inositol was shown to be involved in the maintenance of cell volume homeostasis in human keratinocytes under UV-B irradiation (Warskulat et al., 2004). Finally, UV-B irradiation may also result in *myo*-inositol decrease in rabbit cornea and lens cells, pointing to the interrelation between UV-B response, *myo*-inositol and osmotic regulation (Risa et al., 2005; Tessem et al., 2006). Whether similar mechanisms exist in plants remains unknown. However, IPS1 was shown to be down-regulated under UV-B irradiation in maize (Chen et al., 2001; Content et al., 2003) and *myo*-inositol accumulation was shown to vary upon UV-B exposure (Casati et al., 2011). We suggest that *myo*-inositol deficiency may activate other protective mechanisms against UV. Indeed, we have shown previously in (Meng et al., 2009) that *atips1* mutants grown under short day cultivation (so without cell death) are tolerant to OS. Our hypothesis is that *myo*-inositol deficiency causes a basal stress level that activates protective mechanisms. This priming of plant defense may enhances their tolerance to stress up to a certain threshold, and, beyond this threshold, cell death is induced.

Finally, the increased resistance of *atips1* to OS may be connected not only with the catalytic function of AtIPS1, but also with the involvement of this enzyme in the regulation of gene expression. Indeed the AtIPS1 protein was identified as a interacting partner of the histone methyltransferase *Arabidopsis* Trithorax-Related Protein 5 (ATXR5) (Meng et al., 2009), and can inhibit its activity to control its own expression (Latrasse et al., 2013). This regulatory function may also occur at other loci, possibly including genes involved in OS response. Transcriptomic analysis of gene expression profiles of *atips1* plants grown under long day conditions revealed upregulation of 52 genes potentially involved in the processes associated with OS mediation (Meng et al., 2009), whether this effect is due to direct regulation of their expression by AtIPS1 or to indirect effects caused by modified *myo*-inositol accumulation remains to be established. Enhanced protective effect of NO in plants deficient for AtIPS1 sheds light on the relationship between this enzyme functions and regulation of expression and/or activity of antioxidant systems upon exposure to UV-B in plant cell.

Presented results are a prerequisite for further study of the role of the 1-L-*myo*-inositol-1-phosphate 1 in the mediation of

external abiotic impacts, including UV-B. On this step the interrelations between *myo*-inositol metabolism, NO signaling, and OS mediation became evident under both harmful influence of NO excess (insensitivity of underground tissues of the mutant to nitrosative impact comparing to wild type) and variability of tissues-specific reduction/oxidation answers of the *atips1* and Col-0 plants to different concentrations of NO donor. Higher tolerance to UV-B action and increased receptivity to protective NO effects of *atips1* are the arguments of mentioned interrelation too (summarized in **Figure 6**). Unraveling the underlying molecular mechanisms will be a challenging goal for future investigation.

AUTHOR CONTRIBUTIONS

DL, formulation of the work conception and experimental design. Performing of the experiments, data analysis, and manuscript preparing. CR, obtaining of transgenic plants, data analysis, and interpretation, manuscript preparing, and revising it critically for important intellectual content. AY, experimental design, data analysis, and interpretation, manuscript preparing and final approval of the version to be published. CB, formulation of the work conception, obtaining of transgenic plants, data analysis, and interpretation. YB, contributions to the work conception, data analysis, interpretation of results, and final approval of the version to be published.

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Heme Oxygenase-1 Delays Gibberellin-Induced Programmed Cell Death of Rice Aleurone Layers Subjected to Drought Stress by Interacting with Nitric Oxide

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Cereal aleurone layers undergo a gibberellin (GA)-regulated process of programmed cell death (PCD) following germination. Heme oxygenase-1 (HO-1) is known as a rate-liming enzyme in the degradation of heme to biliverdin IXα, carbon monoxide (CO), and free iron ions (Fe^{2+}). It is a critical component in plant development and adaptation to environment stresses. Our previous studies confirmed that HO-1 inducer hematin (Ht) promotes the germination of rice seeds in drought (20% polyethylene glycol-6000, PEG) conditions, but the corresponding effects of HO-1 on the alleviation of germination-triggered PCD in GA-treated rice aleurone layers remain unknown. The present study has determined that GA co-treated with PEG results in lower *HO-1* transcript levels and HO activity, which in turn results in the development of vacuoles in aleurone cells, followed by PCD. The pharmacology approach illustrated that up- or down-regulated *HO-1* gene expression and HO activity delayed or accelerated GA-induced PCD. Furthermore, the application of the HO-1 inducer Ht and nitric oxide (NO) donor sodium nitroprusside (SNP) not only activated *HO-1* gene expression, HO activity, and endogenous NO content, but also blocked GA-induced rapid vacuolation and accelerated aleurone layers PCD under drought stress. However, both HO-1 inhibitor zinc protoporphyrin IX (ZnPPIX) and NO scavenger 2-(4-carboxyphenyl0-4, 4,5,5-tetramethylimidazoline-l-oxyl-3-oxide potassium salt (cPTIO) reserved the effects of Ht and SNP on rice aleurone layer PCD under drought stress by down-regulating endogenous HO-1 and NO, respectively. The inducible effects of Ht and SNP on *HO-1* gene expression, HO activity, and NO content were blocked by cPTIO. Together, these results clearly suggest that HO-1 is involved in the alleviation of GA-induced PCD of drought-triggered rice aleurone layers by associating with NO.

Keywords: heme oxygenase-1, drought stress, *Oryza sativa*, aleurone layers, programmed cell death, nitric oxide, gibberellin

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INTRODUCTION

Programmed cell death (PCD) plays a critical role in regulating plant development and in protecting from stress. Cereal aleurone layers are an ideal model for studying the effects of different factors on PCD. The aleurone layers, which surround the starchy endosperm of cereal grains, synthesize, and secrete various enzymes to decompose stored nutrients of endosperm, which in turn are utilized for embryo growth during seed germination (Mundy and Rogers, 1986). After completion of secretion, aleurone layer cells undergo PCD, which is regulated by gibberellin (GA) and abscisic acid (ABA; Bethke et al., 1999). Hence, the PCD of aleurone layers is the central event controlling germination; it decides the germination of seeds and supports the early seedlings growth. Previous investigations have declared that the typical morphological features of PCD occurring in the dying cereal aleurone cells include the fusion of small protein storage vacuoles (PSVs) into bigger ones, which eventually result in a single, large, spherical vacuole occupying almost the entire cytoplasmic area (Bethke et al., 1999). This process of developing vacuoles is referred to an vacuolation (Domínguez et al., 2004). In addition, GA not only triggers the metabolic activation of dormant aleurone cells, but also accelerates its PCD (Fath et al., 2000). The reduced ability of GA-treated cells to scavenge reactive oxygen species (ROS) results in the PCD of aleurone layers (Fath et al., 2001). However, to date, studies on the features of aleurone layer cells in the germinating cereal seeds during PCD have mainly focused on barley, whereas those of other cereals including rice have not been investigated.

Nitric oxide (NO) is a downstream signaling factor that is largely involved in auxin-mediated events (López-Bucio et al., 2006), such as promoting lateral root and adventitious root growth (Pagnussat et al., 2002). In addition, NO not only participates in a hypersensitive response (HR), but also prevents cell death (Delledonne et al., 1998; Beligni et al., 2002). In GA-treated barley aleurone layers, NO delays the loss of catalase (CAT) and superoxide dismutase (SOD), thereby, postponing the occurrence of PCD (Beligni et al., 2002). More recently, NO has been shown to alleviate salt-induced oxidative stress in wheat seedlings (Xie et al., 2008), promote seed germination, and growth in collaboration with heme oxygenase-1 (HO-1) under osmotic stress (Liu et al., 2010).

Heme oxygenase (EC 1.14.99.3) is an intracellular enzyme that catalyzes the oxidative degradation of heme into biliverdin IXα (BV), free iron ions (Fe^{2+}), and carbon monoxide (CO; Muramoto et al., 2002; Devadas and Dhawan, 2006; Gohya et al., 2006; Bilban et al., 2008). All three products also have important physiological functions. Biliverdin is subsequently reduced into bilirubin (BR) under the action of biliverdin reductase (Wilks, 2002), and bilirubin is a kind of strong endogenous antioxidant cytoprotectant (Baraňano et al., 2002; Jansen et al., 2010). In recent years, research has suggested that HO-1 possesses very strong antioxidant properties in different resistance to oxidative stress for his two by-products, namely, BV and BR (Shekhawat and Verma, 2010). Fe^{2+}, as one of the products catalyzed by HO-1, induces the ferritin to increase the antioxidant properties of cells (Baraňano et al., 2000). Increasing evidence has confirmed that the effects of HO-1 on plant growth and development mainly depend on the gas signal molecule, CO. CO exerts its physiological function through the cGMP pathway (Xuan et al., 2007). HO and the HO/CO system, which are considered as frontline factors that combat oxidative damage, also play important roles in wheat root growth (Liu et al., 2010).

To date, three types of HO isozymes have been identified. HO-1 is inducible, whereas, HO-2 and HO-3 are constitutively expressed and have very low activity (Ryter et al., 2002, 2006; Kim et al., 2006). Only HO-1 can be induced by a variety of stimuli that provoke oxidative stress such as heme, heme derivatives (hemin and Ht), and non-heme inducers, including heavy metals, salt, H_2O_2, NO, and its releasing compounds. Exposure of rice seeds to 20% PEG, an HO-1 inducer, results in retardation of seed germination inhibition (data not shown). Therefore, HO-1 has been a research of interest because it exerts a variety of beneficial physiological functions in plants. The protection of HO-1 against oxidative damage has been demonstrated in wheat (Huang et al., 2006; Xie et al., 2008; Wu et al., 2011), soybean (Noriega et al., 2004; Yannarelli et al., 2006), *Arabidopsis* (Xie et al., 2011, 2012), and alfalfa (Han et al., 2008). Furthermore, HO-1 also participates in developmental processes in plants such as root formation (Xuan et al., 2008; Cao et al., 2011; Lin et al., 2012) and seed germination (Xu et al., 2011). The effect of auxin on adventitious root formation in cucumber is realized by rapidly activating the activity of HO, which in turn results in the HO product, CO, thereby triggering signal transduction events (Xuan et al., 2008). Enhancement of *HO-1* transcription and HO activities in the antioxidant defense system in soybean leaves subjected to lower levels of cadmium (Cd) stress (Noriega et al., 2004, 2007) or UV-B irradiation (Yannarelli et al., 2006). There is mounting evidence that shows that HO-1 functions by coordinately interacting with NO. Xuan et al. (2012) previously reported that NO located in downstream of the HO-1 inducer hemin promotes cucumber adventitious rooting, whereas Noriega et al. (2007) suggested that NO up-regulated HO-1 transcript levels and enhanced HO activities in soybean leaves.

The role of HO-1 in regulating PCD by GA, NO, and H_2O_2 has been elucidated in wheat (Wu et al., 2011, 2014). However, the role of HO-1 in regulating PCD, particularly the morphological changes involving vacuoles has not been investigated in rice aleurone layers. Moreover, a relationship between HO-1 and NO signaling has not been established. Based on these statements and considering that PCD is a key event of cereal seed germination, we investigated the relationship of HO-1, NO, and GA during PCD in rice aleurone layers subjected to drought stress. The aims of the present study were to elucidate the function of HO-1 in the alleviation of GA-induced PCD in rice aleurone layers and to confirm whether this effect is caused by an interaction with NO.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma (St. Louis, MO, USA) unless stated otherwise. In the present study, 20% polyethylene

glycol-6000 (PEG) was used to mimic drought stress. Ht, a HO-1 inducer, was used at a concentration of 1 μM (dissolved in 0.1 mM NaOH). Zinc protoporphyrin IX (ZnPPIX), an inhibitor of HO-1 (Fu et al., 2011; Xie et al., 2011; Bai et al., 2012; Cui et al., 2012), was used at a concentration of 10 μM (dissolved in 0.1 mM NaOH). 2-(4-carboxyphenyl)-4, 4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO), as a specific NO scavenger, was used at a concentration of 200 μM. NO-specific fluorophore 4,5-diaminofluorescein diacetate (DAF-2DA) was purchased from Calbiochem (San Diego, CA, USA) and used at a concentration of 10 μM (dissolved in 0.01 mM DMSO). Gibberellic acid (GA) was used at a concentration of 50 μM (dissolved in alcohol). Sodium nitroprusside (SNP), a NO donor, was used at a concentration of 200 μM.

Plant Material, Growth Conditions, and Treatments

Seeds of rice (*Oryza sativa* L. cv. You II 128) were sterilized with 0.1% potassium permanganate for 5 min and extensively washed with distilled water. To promote seed germination, the seeds were placed in a Petri dish containing two layers of filter paper that were moistened with sterile water at a constant temperature 25°C for 1 day. The embryos and the ends of the seeds were removed, and then transferred to Petri dishes containing two sheets of filter paper moistened with distilled water for 2 days. Then, under sterile conditions, the aleurone layers were stripped from de-embryonated half-seeds. Isolated aleurone layers were directly incubated in a medium containing 20% PEG alone, or in the absence or presence of 50 μM GA, 1 μM Ht, 200 μM SNP, 10 μM ZnPPIX, and 200 μM cPTIO, at various time points. Layers incubated in water alone were used as control. Each treatment was repeated at least three times, and 30 aleurone layers were used in each replicate.

Quantitative Real-Time PCR (QRT-PCR) Analysis

Total RNA was isolated from 30 pieces of aleurone layers by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). After being isolated, the RNA samples were treated with RNAase-free DNase (TaKaRa, Dalian, China) to eliminate traces of DNA, then the RNA was quantified by using a UV-1800 spectrophotometer (Shimadzu, Japan). Reverse transcription was performed using the PrimeScript RT reagent Kit (TaKaRa, Dalian, China), according to the manufacturer's instructions. DNA-free total RNA (0.5 μg) from different treatment was used for first-strand cDNA synthesis in a 20-μL reaction volume containing 4 μL 5x PrimeScript Buffer 2, 1 μL PrimeScript RT Enzyme Mix, and 1 μL RT Primer Mix. The relative *HO-1* mRNA levels of the rice aleurone layers were quantified by QRT-PCR, using the SYBR Green real-time PCR Master Mix (TianGen, Beijing, China), following the manufacture's procedures. The PCR Master Mix per reaction contained 9 μL of 20x SYBR Green (containing 2.5x Real Master Mix), 4 μL of cDNA, and 0.5 μL of each oligonucleotide primer. The analysis of real-time PCR data was based on the comparative threshold cycle (C_T) method, which involved normalizing against the transcript levels of EF-1α. The sequences of the primers were used for PCR amplification were based on the following: *HO-1* gene (GenBank Accession Number: CA753857) upstream primer 5′-TCAAGGAACAGGGTCACACAA-3′, 5′-CCTCCAGCCGTATGAGCAA-3′ (downstream primer 5 amplified fragment 142 bp): reference gene of EF-1α (GenBank Accession Number: AA753281) upstream primer 5′-ACGGCAAAACGACCAAGAAG-3′, 5′-CAAGAA CGGTGATGTGGTATGG-3′ (downstream primer 5 amplified fragment).

Determination of HO Activity

For extraction of HO, 30 pieces of aleurone layers were homogenized in 15 mL of 25 mM HEPES-Tris cold buffer solution (pH 7.4) containing 250 mM mannitol, 3 mM EDTA, 3 mM EGTA, 250 mM KI, 1 mM DTT, 0.1 $g{\cdot}L^{-1}$ BSA, 10 $g{\cdot}L^{-1}$ polyvinylpyrrolidone (PVP), and 10% glycerol. The homogenates were filtered across four layers of gauze, and the filtrate was centrifuged at 60,000 × *g* for 30 min at 4°C, then the supernatant was used for the determination of HO activity as described elsewhere (Han et al., 2008). The concentration of BV was evaluated using a molar absorption coefficient in 6.25 $mM^{-1}{\cdot}cm^{-1}$ in 0.1 M HEPES-NaOH buffer (pH 7.2) and at a wavelength of 650 nm. One unit of activity (U) was determined by determining the quantity of the enzyme to produce 1 nmol of BV per 30 min. Determination of protein content was determined using the Coomassie brilliant blue method (Bradford, 1976), with bovine serum albumin as standard.

Determination of Cell Viability and Death

Double fluorescence probes FDA and FM4-64 were used to determinate the viability of rice aleurone layers cells (Bethke and Jones, 2001). The layers were stained with 2 $\mu g{\cdot}mL^{-1}$ FDA (20 mM $CaCl_2$) for 15 min, followed by 20 mM $CaCl_2$ to remove background fluorescence, stained with 1 $\mu g{\cdot}mL^{-1}$ FM4-64 (20 mM $CaCl_2$) for 3 min, then with 20 mM $CaCl_2$ to wash away background fluorescence. Images of the aleurone layers were captured with a laser scanning confocal microscope (LSCM, FV1000, Olympus). Four fields of each aleurone layer were randomly selected, and at least three different aleurone layers were measured per treatment. The number of live and dead cells was counted to determine the percentage of viable cells in different fields, and the numbers were averaged for each half-seed.

Detection of NO Content

The aleurone layers were treated with 0.1 M Tris-HCl buffer (pH 7.4) for 30 min, and then incubated with DAF-2DA (10 μM in 0.1M Tris-HCl buffer) in the dark for 2 h. Excess probes were removed by washing with 0.1 M Tris-HCl buffer three times, each for 15 min (Piantadosi, 2002). The distribution and imaging of NO molecules in the aleurone layers were scanned using a LSCM (FV1000, Olympus) at a moderate speed at an excitation wavelength of 488 nm and an emission wavelength of 500–530 nm.

Statistical Analysis

Data were expressed as the mean ± standard error of at least three independent experiments, and statistical significance was estimated using Duncan's multiple test ($P < 0.05$).

RESULTS

HO-1 Inducer and NO Donor Up-Regulated *HO-1* mRNA and HO Activity of Rice Aleurone Layers Subjected to Drought Stress

To assess whether the different levels of HO activity caused by changes in *HO-1* mRNA expression, QRT-PCR was performed. PEG treatment alone significantly inhibited the transcription level of the *HO-1* gene in rice aleurone layers, whereas PEG co-treated with Ht and SNP led to the attenuation of PEG-induced *HO-1* gene expression. The combined treatment of PEG + Ht showed similar results as that observed using PEG + SNP. Compared to the treatments PEG + Ht and PEG + SNP, the PEG + SNP + Ht treatment did not significantly improve the expression of the *HO-1* gene, and no additive effect was observed using Ht and SNP. Similar results were observed in a study on de-etiolation of wheat seedling leaves (Liu et al., 2013). The effects induced by the HO-1 inducer, Ht, and the NO donor, SNP, were, respectively, reversed by ZnPPIX, a specific inhibitor of HO-1, and cPTIO, a scavenger of NO (**Figure 1A**).

Treatments including the NO donor, SNP, the HO-1 inhibitor, ZnPPIX, and PEG attenuated the decrease in HO-1 activity in aleurone layers treated with PEG alone (**Figure 1B**). Treatment using PEG + SNP and PEG + SNP + ZnPPIX for 24 h did not result in any distinct differences in the HO-1 activities of the aleurone layers, whereas these, respectively, increased by 1.95- and 2.58-fold compared to that using PEG alone (**Figure 1B**), which indicates that the HO-1 inhibitor, ZnPPIX, did not prevent the NO effect. Meanwhile, the observed changes in HO-1 activity were in agreement with the detected *HO-1* expression profile.

After treatment with PEG + Ht + cPTIO for 24 h, the relative expression level of *HO-1* and the activity of HO-1 in rice aleurone layers, respectively, decreased by 33.30 and 55.94% relative to that using PEG + Ht (**Figures 1A,B**). Meanwhile, no statistically significant differences between the treatments PEG and PEG + Ht + ZnPPIX were observed. These results indicated that the NO-specific scavenger cPTIO blocked the Ht-induced expression of *HO-1* by eliminating endogenous NO under drought stress. These results thus suggest that HO-1 acts downstream of NO in the rice aleurone layers under drought stress. Xuan et al. (2012) previously showed that HO/CO possibly acts as the downstream molecule of NO during the process of adventitious rooting in cucumber.

HO-1 Increases the Level of Endogenous NO in Rice Aleurone Cells Under Drought Stress

To further elucidate the relationship of upstream and downstream between HO-1 and NO during the PCD of rice aleurone layers subjected to drought stress, DAF-2DA, the specific NO fluorescence probe, was used to label the cells of the cultured rice aleurone layers subjected to various treatments for 24 h. The NO signal of cells was captured by LSCM (**Figures 2A–J**). The NO fluorescent signal was strong in rice aleurone cells under normal cultivation conditions, which clearly indicated that the level of intracellular NO was high (**Figure 2A**). In contrast, barely detectable NO fluorescent signals were observed under drought stress (**Figure 2B**), indicating that the formation of endogenous NO was significantly restrained under drought stress. However, the combined treatments of PEG + Ht (**Figure 2C**) or PEG + SNP (**Figure 2D**) showed stronger fluorescence signals compared to that using PEG alone, indicating that the HO inducer, Ht, induced NO synthesis in the rice aleurone cells under drought stress.

The combination of the NO donor, SNP, with the HO-1 inhibitor, ZnPPIX, effectively alleviated the decrease in the level of endogenous NO under drought stress (**Figure 2E**); therefore, the intracellular fluorescence intensity was distinct within 24 h of treatment, and was similar to that observed with PEG + SNP. These results indicated that the HO-1 inhibitor, ZnPPIX, did not reduce the synthesis of NO, which was induced by the NO donor, SNP, under drought stress. PEG + SNP + Ht (**Figure 2F**) treatments including the NO scavenger such as PEG + Ht + cPTIO and PEG + SNP + cPTIO effectively prevented NO formation, which was elicited by the HO-1 inducer, Ht, and the NO donor, SNP, in the rice aleurone layers after 24 h of treatment (**Figures 2G,H**). These findings indicated that the NO-specific scavenger, cPTIO, blocked the forming of intracellular NO, which was induced by HO-1 and SNP in the aleurone layers.

GA-Induces the Down-Regulation of *HO-1* Gene Expression and Activity Level in Rice Aleurone Layers Subjected to Drought Stress

By fluorescent QRT-PCR relative analysis, we compared the results with those using distilled water (C, control), the PEG alone treatment significantly inhibited the expression level of *HO-1* by 44%, and exogenous GA treatment also inhibited the expression level of *HO-1* by 27%, but the treatment combined GA with PEG decreased by 33.80% compared to that observed in the GA alone treatment (**Figure 3A**). The treatments PEG + GA + Ht and PEG + GA + SNP partly eased the reduction in the *HO-1* expression level, compared to the treatment with PEG + GA, and respectively, increased by 29.79 and 42.55% (**Figure 3A**). The expression levels of the *HO-1* gene showed no significant differences among treatments PEG, PEG + GA, and PEG + GA in the presence of exogenous HO-1 inhibitor or NO scavenger, and changes in HO activity changes induced by these reagents were identical (**Figure 3B**). The HO-1 activities of rice aleurone layers treated with exogenous GA alone or PEG + GA were significantly inhibited ($P < 0.05$ or $P < 0.01$; **Figure 3B**).

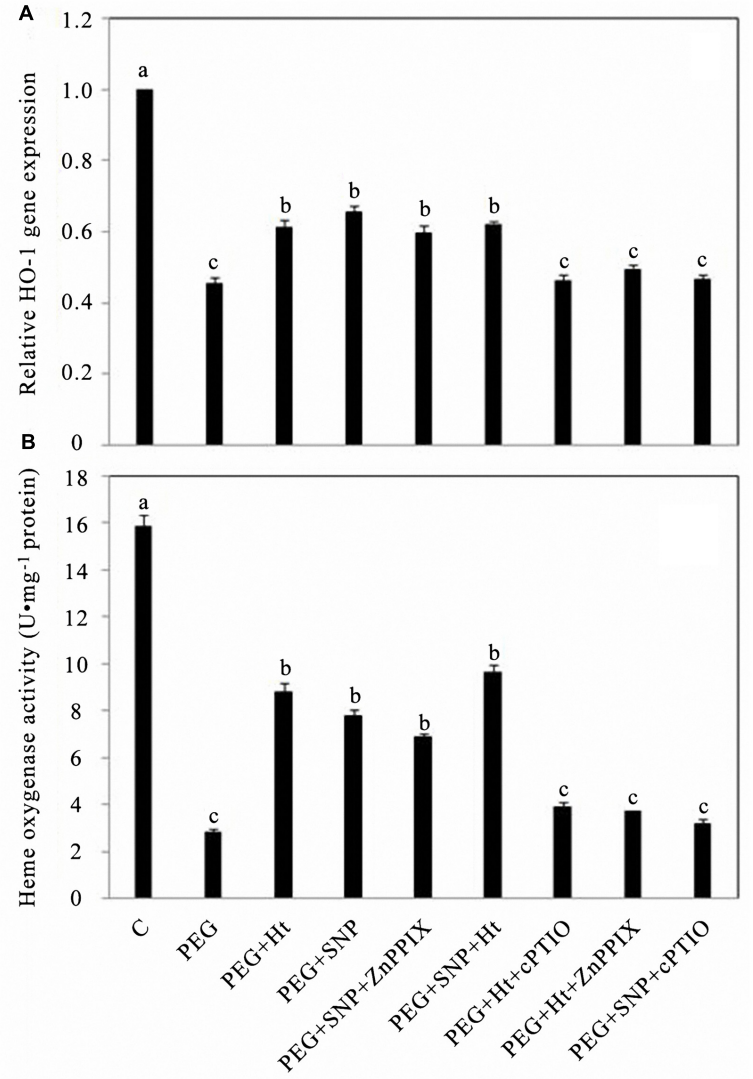

FIGURE 1 | Hematin (Ht), sodium nitroprusside (SNP), zinc protoporphyrin IX (ZnPPIX), and 2-(4-carboxyphenyl0-4, 4, 5, 5-tetramethylimidazoline-l-oxyl-3-oxide potassium salt (cPTIO) up- or down-regulated *HO-1* gene expression (A) and HO activity (B) in rice aleurone layers subjected to drought stress. Rice aleurone layers were treated with or without 20% (polyethylene glycol-6000) PEG, 1 μM Ht, 200 μM SNP, 10 μM ZnPPIX, 200 μM cPTIO individually or in combination for 24 h. A sample with distilled water was used as control (C). Measurement of *HO-1* transcript levels in the rice aleurone layers was conducted by real-time fluorescence quantitative PCR assay **(A)**, HO activities were also detected **(B)**. Mean values were calculated from at least three independent experiments, bars with different letters indicate statistically significant differences at the 0.05 level, using Duncan's multiple test.

Both treatments PEG + GA + Ht and PEG + GA + SNP partly, respectively, raised the HO-1 activities inhibited by the treatment PEG + GA by 2.34- and 2.04-fold (**Figure 3B**). The treatment of HO-1 inhibitor, ZnPPIX, being added into PEG + GA + Ht, compared to the treatment PEG + GA + Ht, significantly reduced the HO-1 activity that was induced by Ht, thus indicating that the response was reserved by ZnPPIX (**Figure 3B**). Because of the participation of NO scavenger cPTIO, the treatment PEG + GA + SNP + cPTIO significantly decreased the effects of NO on raising the HO-1 activity of aleurone cells treated with PEG + GA (**Figure 3B**).

GA, HO-1, and NO Promoted or Delayed PCD in Rice Aleurone Layers Under Drought Stress

The aims of the present study were to examine, whether HO-1 performs a crucial function in the induction of PCD in germinating rice aleurone layers and to confirm whether this effect is caused by its interaction with NO and GA signaling.

FDA and FM4-64 are fluorescence dyes commonly used to identify cell viability, wherein live cells emit green fluorescence and dead cells red fluorescence. This facilitates in distinguishing between live and dead cells in the aleurone layers.

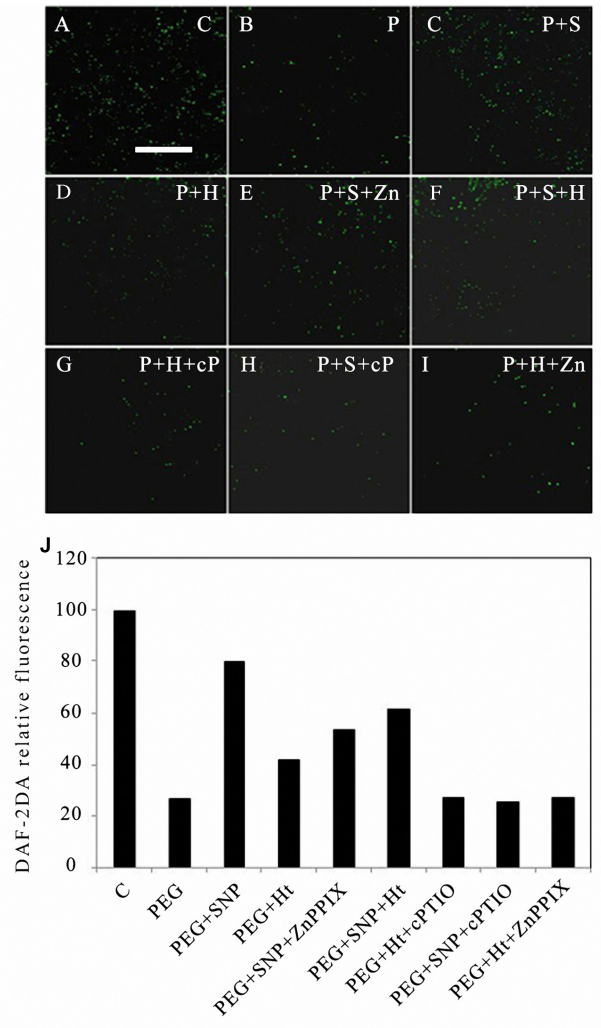

FIGURE 2 | Ht, SNP, ZnPPIX, and cPTIO raised or reduced endogenous NO content in rice aleurone layers under drought stress. Rice aleurone layers were treated with or without 20% PEG (P), 1 μM Ht (H), 200 μM SNP (S), 10 μM ZnPPIX (Zn), and 200 μM cPTIO (cP) individually or in combination for 24 h. A sample with distilled water was used as control (C). After various treatments, the aleurone layers were, respectively, stained with DAF-2DA, and then thoroughly washed to removal excess dye and immediately observed under a LSCM. Images of the distribution of NO in fluorescently labeled aleurone cells were captured **(A–I)**. The relative DAF-2DA fluorescence intensity in the corresponding aleurone layers was also established **(J)**. Scale bar, 100 μm. Mean values were calculated from at least three independent experiments, bars with different letters indicate statistically significant differences at the 0.05 level, according to Duncan's multiple test.

FDA and FM4-64 dual fluorescent staining combined with LSCM showed that the number of dead cells gradually prolonged the incubation time of rice aleurone layers subjected to various treatments (**Figures 4A–X**). Dead cells did not appear in rice aleurone layers within 24 h of distilled water culture (C, control), and most of cells showing green fluorescence after 48 h of incubation were still alive (**Figures 4A–C**). After treating aleurone layers with exogenous GA for 24 h, some cells died, and after 48 h, more than half of the cells were dead (**Figures 4D–F**). On the other hand, PEG and co-treatment with PEG and GA accelerated the death rate of aleurone cells; as early as 12 h after treatment, cells were dead, and within 48 h, almost all cells were dead in the aleurone layer (**Figures 4G–L**). While the treatments containing the HO-1 inducer or NO donor elevated the survival rate of the aleurone layer cells at 12, 24, and 48 h, even after 48 h, 35.67 and 48.33% of the cells remained alive (**Figures 4M–R**). On the other hand, treatments PEG + GA + Ht + ZnPPIX and PEG + GA + SNP + cPTIO showed no distinct differences at each time point, almost all cells of the aleurone layer were dead within 48 h of treatment (**Figures 4S–X**). These findings illustrated that the respective inhibitor and scavenger reversed the effects of HO-1 and NO on delaying PCD.

A coordinate curve of cell survival rate and time of various treatments was constructed (**Figure 4Y**). The aleurone layers showed a 90% cell survival rate within 48 h of distilled water treatment, whereas that treated with exogenous GA alone decreased to 45% after 24 h of drought stress, and almost no live cells were observed after 48 h. The cell survival rates of aleurone layers remained at 38 and 33% after 48 h of incubation in PEG + GA + Ht and PEG + GA + SNP, respectively, whereas when the treatments PEG + GA + Ht + ZnPPIX and PEG + GA + SNP + cPTIO were applied to the aleurone layer for 48 h, the cells remained alive (**Figure 4Y**), indicating that HO-1 and NO delayed the GA induction the cell death in rice aleurone layers subjected to drought stress.

GA Accelerates the Development of a Large Central Vacuole in Aleurone Cells Under Drought Stress, and HO-1 and NO Slows the Effect of GA

The large central vacuole of aleurone cells is the prelude to PCD during cereal seed germination (Bethke et al., 1999). Therefore, our next goal was to explore, whether the vacuolation was associated with the PCD of aleurone layers in germinating rice seeds subjected to drought stress.

By exploring morphological changes of aleurone cells during PCD under normal cultured conditions, we determined that the morphological changes of PSVs were significant in the aleurone cells. Therefore, based on the morphological changes in PSVs, the cell death process of rice aleurone layers could be determined. From 0 to 1 day of culture, aleurone cells were hexagonal, with nearly uniform wall thickness, and with spherical-shaped aleurone grains (**Figure 5A**, arrow). When aleurone layers were cultured from 2 to 6 days, the PSVs of aleurone cells began to merge into larger vesicles (**Figures 5B–E**, arrows) accompanied by vacuolation, a large central vacuole appeared after 7 days (**Figure 5F**, arrow), and then the large central vacuole deformed and elongated, and integrity of the vacuole membrane disappeared after 8 days (**Figure 5G**, arrow). Finally, vacuole rupture (**Figure 5H**, arrow) resulted in protoplast dissolution, followed by shrinking into a ball after 10 days (**Figure 5I**, arrow).

In the GA alone treatment, several small PSVs were observed in the aleurone cells within 1/2 day (**Figure 6A**, arrow). However,

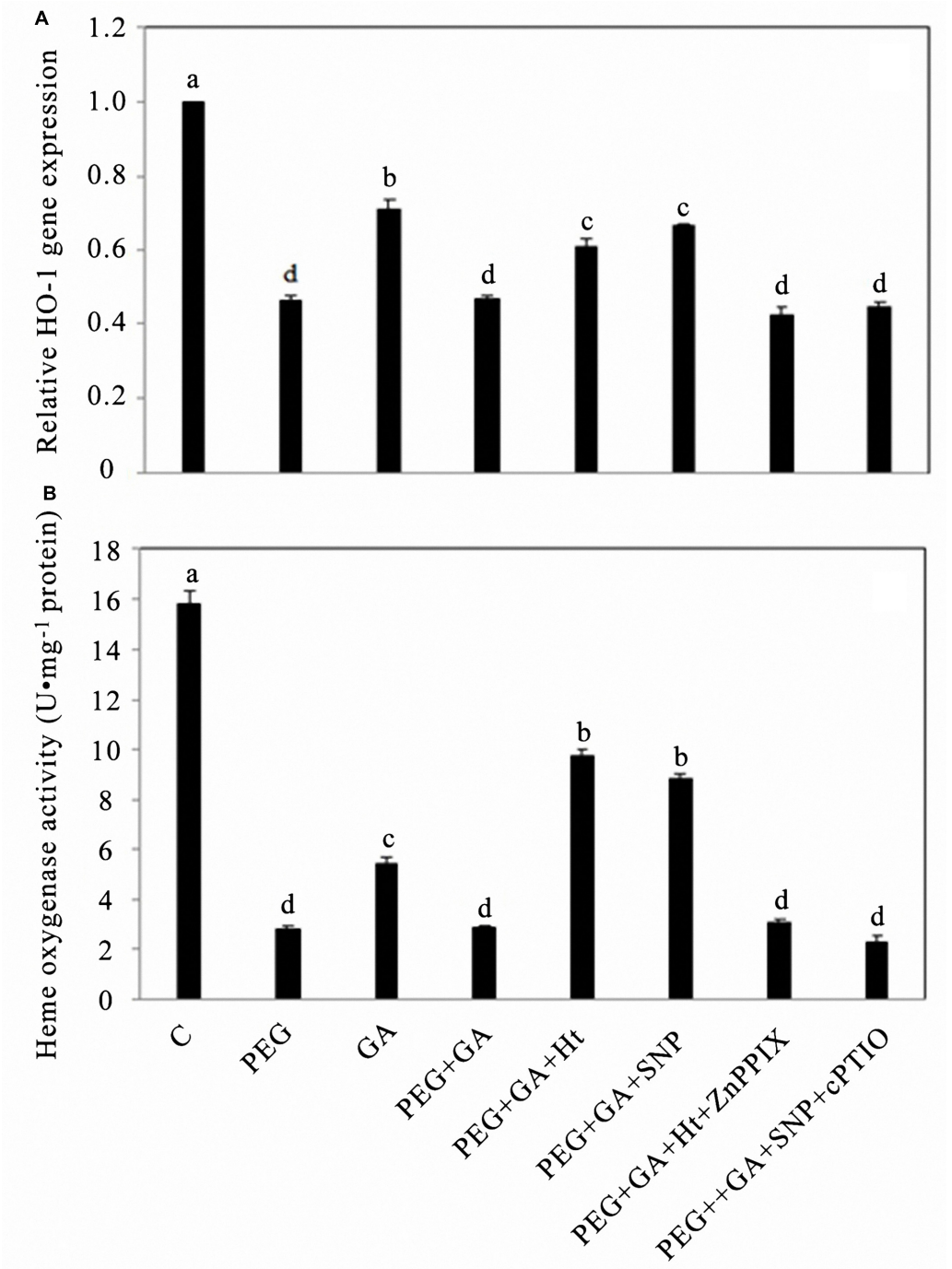

FIGURE 3 | Ht, SNP, ZnPPIX, and cPTIO up- or down-regulated the expression of *HO-1* and the activity of HO in GA-induced rice aleurone layers subjected to drought stress. Aleurone layers treated with or without 20% PEG, 50 μM GA, 1 μM Ht, 200 μM SNP, 10 μM ZnPPIX, and 200 μM cPTIO alone or in combination for 24 h. A sample with distilled water treatment was used as control (C). The transcript levels of *HO-1* analyzed by real-time fluorescence quantitative PCR at 24 h. The expression levels of the *HO-1* gene are presented as values relative to that of the control **(A)**. Meanwhile, the corresponding HO activity **(B)** was determined after different treatments for 24 h. Mean values were calculated from at least three independent experiments, bars with different letters indicate significant differences at the 0.05 level, according to Duncan's multiple test.

the PSVs began to fuse and formed into several bigger vacuoles in GA-treated aleurone cells after incubation for 1 day (**Figure 6B**, arrow), and then these larger PSVs fused to form a large central vacuole after 2 days (**Figure 6C**, arrow). The large central vacuole elongated and deformed, and the deformed large central vacuole showed an unclear boundary after 3 days (**Figure 6D**,

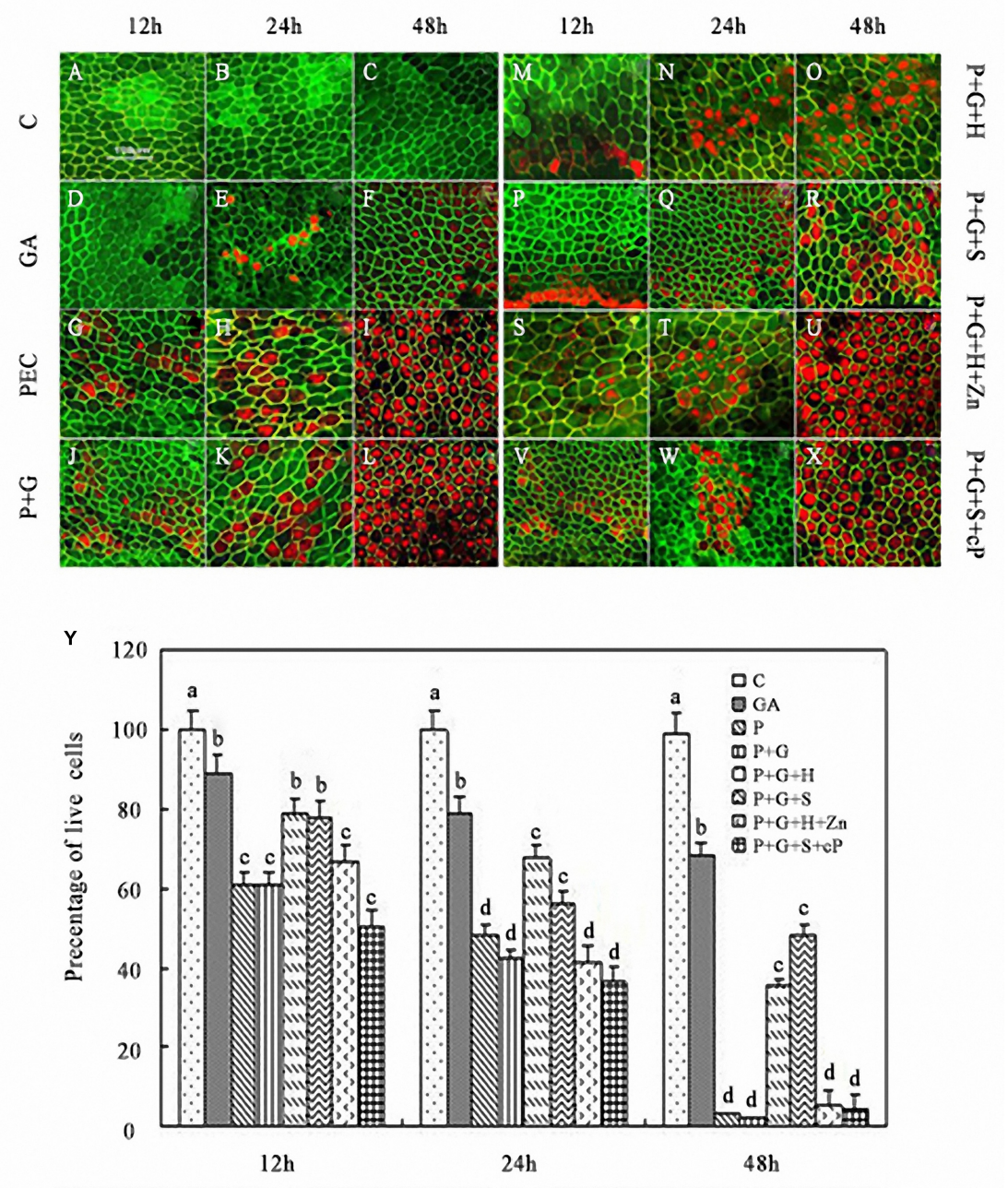

FIGURE 4 | Increase or decrease in *HO-1* gene expression and HO activity related to the acceleration or delay of GA-triggered PCD in rice aleurone layers under drought stress. Aleurone layers were incubated in FDA (green, live cells) and FM-4-64 (red, dead cells) prior to image capture. Aleurone layers treated with or without 20% PEG (P), 50 μM GA (G), 1 μM Ht (H), 200 μM SNP (S), 10 μM ZnPPIX (Zn), and 200 μM cPTIO (cP) alone or in combination for 12, 24, and 48 h, respectively. A sample with distilled water treatment was used as control (C). Images of cells showing fluorescently labeled aleurone layers were captured **(A–X)**. Cell survival rate was also quantified **(Y)** at 12, 24, and 48 h. Scale bar, 100 μm. Mean values were calculated from at least three independent experiments, bars with different letters indicated significant differences at the 0.05 level, according to Duncan's multiple test.

arrow), and finally, protoplasts appeared shrunken within cells after 4 days (**Figure 6E**, arrow). The death process of aleurone cells was earlier at 6 days compared to cells cultured under normal conditions. These results confirmed that exogenous GA significantly accelerates the vacuolation processes in aleurone cells.

Compared to the GA alone treatment, the PEG and PEG + GA treatments hastened the occurrence of the large central vacuole. The PSVs of aleurone cells treated with PEG and PEG + GA were larger than those treated with GA after 1/2 day (**Figures 6F,I**, arrows), exhibiting a large central vacuole with distinct deformation after 1 day (**Figures 6G,J**, arrows) and

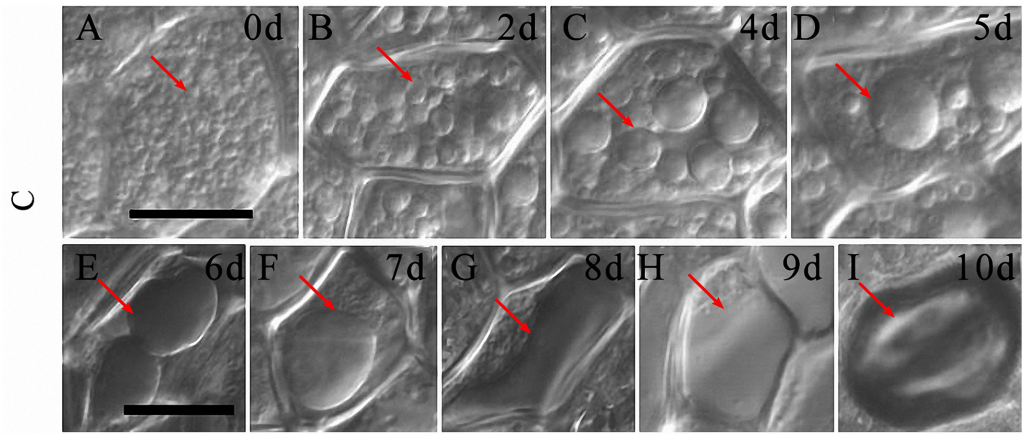

FIGURE 5 | The vacuolated process of aleurone cells treated with distilled water. (A–I) Vacuoles of aleurone cells treated with distilled water at 0, 2, 4, 5, 6, 7, 8, 9, and 10 days. The scale is 20 μm.

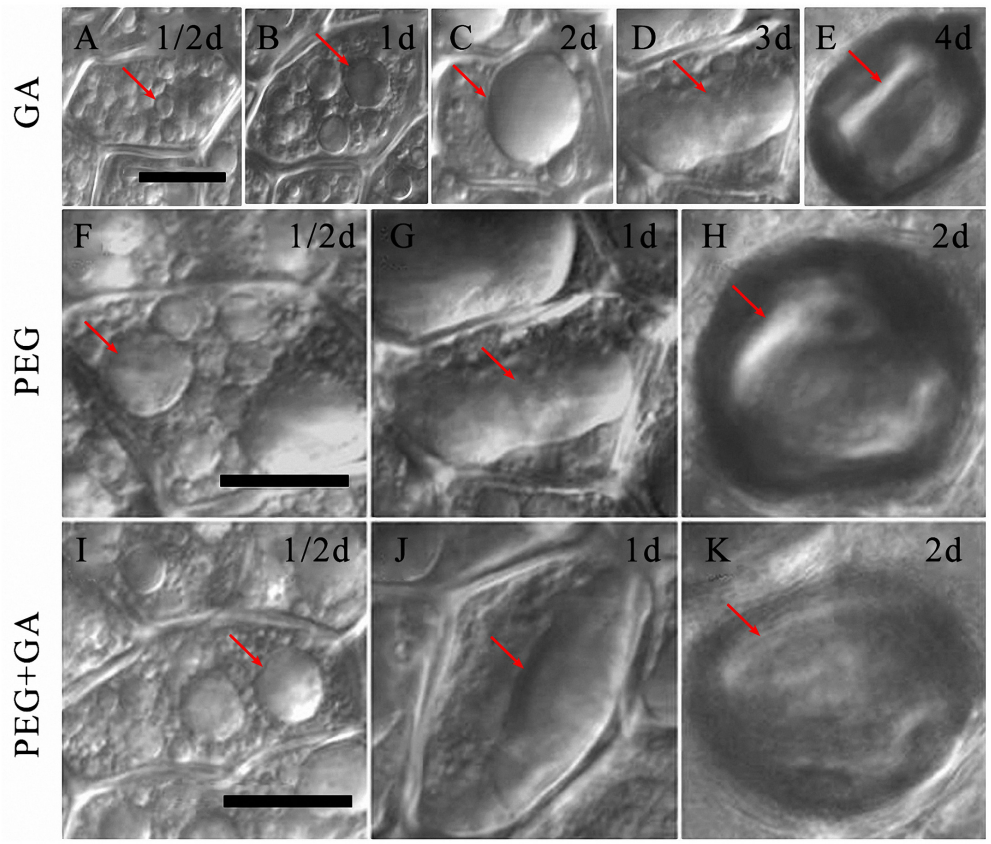

FIGURE 6 | Gibberellin (GA) co-treated with PEG accelerated the vacuolated process of aleurone cells. (A–E) Vacuoles in aleurone cells treated with 50 μM GA at 1/2, 1, 2, 3, and 4 days. **(F–H)** Vacuoles in aleurone cells treated with 20% PEG for 1/2, 1, and 2 days. **(I–K)** Vacuoles in aleurone cells treated with 20% PEG + 50 μM GA for 1/2, 1, and 2 days. The scale is 20 μm.

only after 2 days, presented the phenomenon of cell protoplasts mixing together and shrinking (**Figures 6H,K**, arrows). The results certified that drought stress speeds up the formation and rupture of large central vacuoles in GA-inducing aleurone cells.

When the HO-1 inducer and NO donor were, respectively, added to PEG + GA, a large central vacuole of aleurone cell was not observed at 1 day (**Figures 7A,E**) and 2 days (**Figures 7B,F**). A large central vacuole did not emerge until

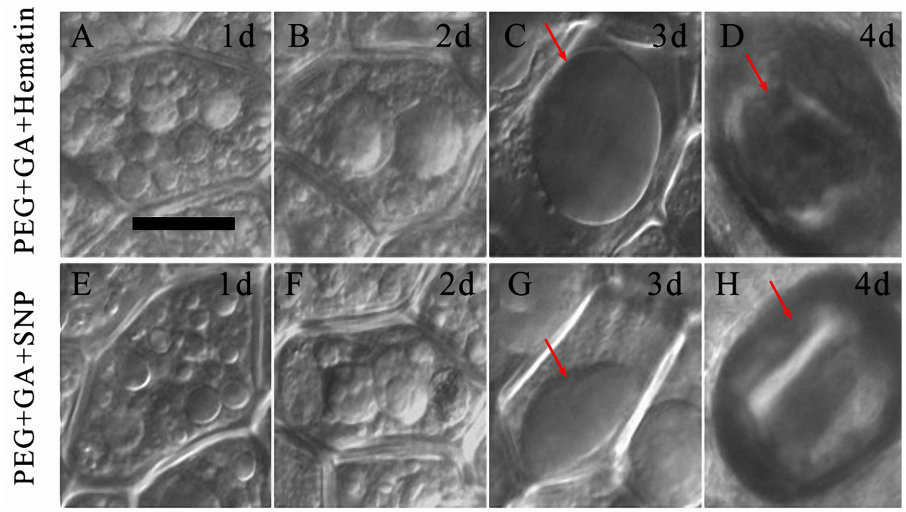

FIGURE 7 | Ht and SNP delayed vacuolation in aleurone cells treated with PEG plus GA. (A–D) Vacuoles of aleurone cells treated with 20% PEG + 50 μM GA + 1 μM Ht at 1, 2, 3, and 4 days. **(E–H)** Vacuoles in aleurone cells treated with 20% PEG + 50 μM GA + 200 μM SNP for 1, 2, 3, and 4 days. The scale is 20 μm.

3 days of the PEG + GA + Ht and PEG + GA + SNP treatments (**Figures 7C,G**, arrows), cell protoplast shrinking was only observed after 4 days (**Figures 7D,H**, arrows), which was then followed by cell death. The occurrence of large central vacuoles was observed two days later in both PEG + GA + Ht and PEG + GA + SNP treatments compared to that in the PEG + GA treatment (**Figures 6I–K**). These results suggest that HO-1 and NO delayed the occurrence of large central vacuoles in GA-induced aleurone cells subjected to drought stress. Therefore, HO-1 and NO contribute to the delay in GA-induced aleurone layers PCD during drought stress.

DISCUSSION

In our previous study, HO-1 inducer Ht differently up-regulated the activities of SOD, CAT, and peroxidase (POD), resulting in the apparent decrease of membrane permeability and malondialdehyde (MDA) content. These results suggested that HO-1 alleviates drought-induced oxidative damage in rice seeds germination (data not shown). Increasing evidence demonstrated that endogenous ROS, particularly H_2O_2, play a critical role in regulating the PCD of cereal aleurone layers (Bethke and Jones, 2001; Wu et al., 2011). Antioxidants and ROS scavengers such as butylated hydroxytoluene (BHT), dithiothreitol (DTT), and ascorbic acid (AsA) not only delayed PCD, but also mimicked the effect of HO-1 inducer haematin on up-regulating HO-1. In addition, haematin blocked the decrease of GA-induced ascorbate peroxidase (APX) and CAT activities, and then resulted in the decrease of H_2O_2 level, and these effects were reversed by HO-1 inhibitor ZnPPIX (Wu et al., 2011). The up-regulation of HO-1 would contribute to the capability of HO, act as a potent antioxidant enzyme, eliminating ROS and protecting cells from oxidative stress in wheat aleurone layers (Wu et al., 2011, 2014), which has previously been confirmed in plants (Noriega et al., 2004; Han et al., 2008). The present study showed that the up-regulation of HO contributed to the delay in PCD in rice aleurone layers induced by GA under drought stress, the similar results were obtained by Wu et al. (2011, 2014). Above mentioned results revealed that HO-1 function in the PCD of cereal aleurone layers is dependent on enhancing the activities of antioxidant enzymes.

Mounting evidence has confirmed that HO-1 and NO play important physiological functions in plants, such as improving resistance and promoting seed germination. After treatment with Ht, the gene transcription and activity level of HO-1 were up-regulated, as well as alleviated Cd-induced oxidative damage in alfalfa root tissue (Han et al., 2008). NO, as an antioxidant, reduced the accumulation of ROS, and alleviated the damage caused by oxidative stress, thereby enhancing the adaptability of plants to abiotic stress (He et al., 2014). In addition, exogenous NO donor SNP induced the up-regulation of HO-1 (Noriega et al., 2007; Xuan et al., 2008; Santa-Cruz et al., 2010), and NO prolonged the life of barley aleurone cells treated with GA (Beligni et al., 2002). The aleurone layers of cereal grains undergo PCD, which is regulated by GA following germination (Domínguez et al., 2004). By exploring the relationship between HO-1 and NO during PCD in rice aleurone layers, we discovered that the HO-1 inducer Ht and NO donor SNP up-regulated the gene expression and activity of HO-1 in the rice aleurone layers under drought stress, but their effects were, respectively, reversed by specific inhibitor ZnPPIX and scavenger cPTIO. Simultaneously, the effect of HO-1 was blocked by NO scavenger cPTIO, whereas HO-1 activity inhibitor ZnPPIX did not block the effect of NO on the up-regulation of *HO-1* gene expression and activity of aleurone layers (**Figures 1A,B**). In addition, treatments using HO-1 inducer or NO donor resulted in an increase in endogenous NO content in aleurone cells under

drought stress (**Figures 2A,B**). Similar results were observed using SNP and Ht, which accelerated NO emission in etiolated wheat seedling leaves (Liu et al., 2013). Further experiments showed that the NO scavenger repressed the action of the HO-1 inducer Ht and NO donor SNP. However, the HO-1 inhibitor ZnPPIX was unable to block the effect of the NO donor of increasing the level of endogenous NO (**Figures 2A,B**). Furthermore, compared to the Ht alone-treated sample, ZnPPIX and cPTIO markedly reduced the Ht-induced up-regulated NO level, suggesting that HO-1-mediated NO production is a major source of endogenous NO in aleurone layers. Based on these results, we deduced that there is a link between HO-1 and NO in GA-induced PCD in rice aleurone layers subjected to drought stress. Similarly, SNP- and Ht-triggered up-regulation of HO-1 was observed in soybean seedlings (Noriega et al., 2007; Santa-Cruz et al., 2010) and wheat aleurone layers (Wu et al., 2011). Li et al. (2015) inferred that HO-1 acting downstream of NO signaling was involved in β-CDH-induced lateral root formation in tomato. A different conclusion was also drawn in that NO acted downstream of HO-1, wherein it is involved in hemin-induced cucumber adventitious rooting (Xuan et al., 2012). Another study also showed that HO stimulated rooting of mung bean hypocotyl cuttings upstream of NOS/NO (Xu et al., 2006).

The aims of the present study were to examine whether HO-1 performs a crucial function in responding to the GA-induced PCD of germinating rice aleurone layers and to confirm whether this effect is caused by its interaction with NO. Previous studies have confirmed that GA initiated PCD in the aleurone layers of wheat (Kuo et al., 1996) and barley (Wang et al., 1996; Bethke et al., 1999). In the present study, GA and PEG, and PEG co-treated with GA significantly reduced the gene expression and activity of HO-1 (**Figures 3A,B**), thereby accelerating PCD in aleurone layers (**Figures 4D–L**), whereas the HO-1 inducer Ht and NO donor SNP alleviated the GA-induced decline in *HO-1* gene expression and activity in rice aleurone layers subjected to drought stress (**Figures 3A,B**), improved cell survival rate, and delayed the occurrence of GA-induced aleurone layer PCD in the germinating rice seeds under drought stress (**Figures 4M–R**). The effects of the HO-1 inducer and NO donor SNP were, respectively, reversed by the HO-1 inhibitor ZnPPIX and NO-specific scavenger cPTIO, whereas PCD in rice aleurone layers was accelerated (**Figures 4S–X**). A previous study suggested that the HO inducer haematin increases HO transcription and activity, and inhibits PCD of wheat aleurone layers, whereas the HO inhibitor ZnPPIX reverses the effect of haematin and contributes to cell death (Wu et al., 2011). These results ensured that intracellular HO plays a major role in postponing the PCD of aleurone layers subjected to drought stress.

The vacuole plays a crucial role in the PCD of plants (Gucciardi et al., 2004; Gadjev et al., 2008; Xiao et al., 2009; Hara-Nishimura and Hatsugai, 2011), wherein only highly vacuolated cells undergo PCD in plants (Bethke and Jones, 2001). Therefore, we wanted to know, whether the degree of vacuolation is associated with PCD in rice aleurone layers. In the present study, we determined that variation in vacuole morphology of aleurone cells sequentially occurred under normal culture conditions; it took 7 days for the large central vacuole to emerge (**Figures 5A–F**). Subsequently, the large central vacuole lost its distinct boundary and in turn underwent deformation, thereby resulting in tonoplast rupture (**Figures 5G–I**). However, GA increased the rate of vacuolation of PEG-induced aleurone layers (**Figures 6I–K**). Therefore, a large number of cells in the aleurone layers were dead (**Figure 4L**). Interestingly, HO-1 inhibitor ZnPPIX and NO scavenger cPTIO mimicked the effect of GA-promoted vacuolation and PCD, and HO-1 inducer Ht and NO donor SNP delayed the appearance of large central vacuoles (**Figures 7C,G**), ultimately resulting in the delay in the onset of PCD in rice aleurone layers subjected to drought stress. Therefore, we speculated that HO-1 and NO are involved in postponing GA-induced vacuolation of aleurone cells in rice under drought stress by preventing the formation of large central vacuoles.

In the present study, we showed that up-regulating HO alleviates GA-induced PCD in rice aleurone layers subjected to drought stress. A similar finding involving the up-regulation of HO that results in a delay in PCD in wheat has been earlier described (Wu et al., 2011).

Previous studies have confirmed that vacuoles are essential in initiating PCD in plants, and vacuole membrane rupture is a critical step in plant PCD (Higaki et al., 2011). However, previous studies did not determine, whether changes in *HO-1* gene expression affected the process of vacuolation and PCD. Therefore, we investigated the relationship of up- or down-regulation of HO-1, vacuolation, and GA-induced PCD progression in rice aleurone layers subjected to drought stress. The results indicated that GA, the HO-1 inhibitor, and the NO scavenger down-regulate the *HO-1* mRNA level and HO activity, which in turn increases the rate of vacuolation and PCD. However, the HO-1 inducer and NO donor slowed down the process of vacuolation and PCD by up-regulating *HO-1* gene expression and HO activity.

In summary, we present evidence that suggest that GA regulates the expression of HO-1 in germinating rice aleurone layers subjected to drought stress. The level of *HO-1* gene expression and HO activity plays an important role in ascertaining the process of GA-induced PCD in response to drought stress. HO-1 and NO modulate each others function in aleurone layers, similar to that in the NO donor SNP, wherein Ht-driven HO-1 promotes the level of endogenous NO. Correspondingly, the enhanced NO triggers the up-regulated expression and activity of HO-1, whereas the NO inhibitor cPTIO down-regulated the expression and activity of HO-1. Therefore, the observed mutual induction effects indicate that there might be an inseparable relationship between HO-1 and NO in delaying the PCD of GA-induced rice aleurone layers subjected to drought stress. However, the PCD of cereal aleurone layers is a complex event, and it is unable to be elucidated clearly only depending on the evidence of biochemistry, cell morphology, and pharmacology. Therefore, future studies aim to investigate the HO, NO, and GA signal transduction pathways and the molecular mechanism in the PCD of rice aleurone layers subjected to drought stress by combining with molecular methods.

AUTHOR CONTRIBUTIONS

HC designed the experiment. HC and HW wrote the paper. HW carried out fluorescence quantitative RT-PCR. YZ carried out cell morphology observation and subcellular localization. JL helped in enzyme activity determination. HC and HZ helped in drafting the manuscript.

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Citrus Plants: A Model System for Unlocking the Secrets of NO and ROS-Inspired Priming Against Salinity and Drought

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Plants treated with chemical compounds can develop an enhanced capacity to resist long after being subjected to (a)biotic stress, a phenomenon known as priming. Evidence suggests that reactive oxygen species (ROS) and reactive nitrogen species (RNS) coordinately regulate plant stress responses to adverse environmental conditions; however, the mechanisms underlying this function remain unknown. Based on the observation that pre-exposure of citrus (*Citrus aurantium* L.) roots to the NO donor sodium nitroprusside (SNP) or to H_2O_2 prior to NaCl application can induce acclimation against subsequent stress we characterized the changes occurring in primed citrus tissues using several approaches. Herein, using this experimental model system, we provide an overview of our current knowledge of the possible mechanisms associated with NO and H_2O_2 priming to abiotic stresses, particularly concerning salinity and drought. The data and ideas presented here introduce six aspects of priming behavior in citrus under abiotic stress that provide knowledge necessary to exploit priming syndrome in the context of sustainable agriculture.

Keywords: abiotic stress, drought, priming, proteins, salinity

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INTRODUCTION

Environmental stress factors, such as drought and salinity, strongly affect plant growth and pose a growing threat to sustainable agriculture (Golldack et al., 2014). This has become a hot issue due to concerns about the effects of climate change on plant resources, biodiversity and global food security (Ahuja et al., 2010). Consequently, understanding the mechanisms underlying plant abiotic stress acclimation helps us to develop fruitful new agricultural strategies (Beckers and Conrath, 2007). Evidence suggests that plants are capable of inducing some stress "memory," or "stress imprinting" following a first stress exposure that leads to acclimation to a later (a)biotic stress. Through priming (also known as hardening), plants are able to induce responses to a range of stresses, providing low-cost protection in relatively high stress-pressure conditions (Borges et al., 2014). Despite priming phenomena have previously been widely described under biotic stress (Prime-A-Plant Group et al., 2006; Conrath et al., 2015) and in the invigoration of seeds (Rajjou et al., 2012), the mechanisms of long-lasting priming are still unclear, notably under abiotic stress (Tanou et al., 2012b). It has been suggested that hormone-dependent pathways and availability of signal transduction proteins along with epigenetic mechanisms, such as histone modifications and DNA

methylation, are involved in priming against abiotic stress (Bruce et al., 2007; Conrath, 2011). Recently, Jiménez-Arias et al. (2015) showed that *Arabidopsis* seed-based priming against salt stress involves epigenetic changes (DNA hypomethylation) in genes controlling proline metabolism. In this regard, it has been proposed that priming stimulates salicylic acid (SA), abscisic acid (ABA), and jasmonic acid (JA) signaling that could facilitate the transcriptional induction of defense genes and epigenetic changes; this *trans*-generational induced resistance is elicited by the RNA-directed DNA methylation (RdDM) pathway, which triggers heritable changes in DNA methylation that can direct priming-inducing chromatin modifications at defense gene promoters (Pastor et al., 2013).

Almost all abiotic stressors generate reactive oxygen species (ROS) and reactive nitrogen species (RNS), resulting in oxidative and nitrosative stress in plants (Molassiotis and Fotopoulos, 2011). It has been increasingly evident that RNS (in the form of nitric oxide, NO) and ROS (in the form of H_2O_2) play important roles in priming phenomena in various annual plants (Del Río, 2015). Nevertheless, a priming approach has a great potential if studied in non-model long-lived fruit trees species (Molassiotis et al., 2010). Citrus is widely cultivated in Mediterranean-type ecosystems where climatic change is expected to amplify drought and salinity stress (Gordo and Sanz, 2010). Basal resistance by itself is too weak to protect citrus against environmental stimuli, since it is sensitive to oxidative and nitrosative stress induced by various abiotic stress conditions (Ziogas et al., 2013). Hence, the possible implications of NO and H_2O_2 in the acclimation of citrus plants to adverse environmental conditions, as well as the interactions between the two molecules, were studied. In this regard, we initially documented at physiological level that NO and H_2O_2 are able to induce priming against salt stress by pre-treating the roots of sour orange (*Citrus aurantium* L.) seedlings either with the NO donor sodium nitroprusside (SNP) or with H_2O_2 prior to NaCl application (see Tanou et al., 2009b). Using this experimental system, the mechanisms by which citrus plants respond to salinity were investigated in order to gain a wide understanding of oxidative- and nitrosative-associated priming in plants. In this review, we summarize our current knowledge of the possible mechanisms associated with NO- and H_2O_2-induced salinity and drought acclimation in citrus plants. Overall, this approach reveals the following six aspects regarding the mechanism of NO- and H_2O_2-associated priming events in citrus against abiotic challenges.

NO AND H_2O_2-ORIGINATED PRIMING IS ASSOCIATED WITH INDUCED ANTIOXIDANT ACTIVITY

One of the mechanisms actively employed by primed plants to survive under abiotic stress is the induction of the antioxidant defense system (Hossain et al., 2015). In citrus plants it was evidenced that pre-treatments with NO and H_2O_2 prior to NaCl stress induced antioxidative defense-related enzymatic activity [e.g., superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR)] (Tanou et al., 2009b) and mRNA expression [(eg., Cu/Zn-SOD, Mn-SOD and Fe-SOD, xanthine oxidoreductase (XO), and alternative oxidase (AOX)] (Tanou et al., 2012a). Another antioxidant function of NO arises from the observation that NO protects citrus leaves from DNA strand cleavage caused by hydroxyl radical $^{\bullet}OH$ produced following NaCl application (Tanou et al., 2009b), thereby avoiding oxidative damage induced by most harmful ROS. It has been proposed that NO protects from $^{\bullet}OH$-stimulated oxidative stress in two possible ways: directly by scavenging ROS, and indirectly by NO-mediated induction of ferritin proteins that contributes to diminishing free-Fe^{2+} levels and Fenton-type reactions within the organelles (Martin et al., 2009). It had been demonstrated that $^{\bullet}OH$ effectively targeted DNA methylation and regulatory genes (Shen et al., 2014), implying that NO may have an important role in priming process, at least in part, by DNA methylation-based epigenetic modifications.

THE PRIMING ACTION OF NO AND ROS IS LONG–DISTANCE AND LONG-LASTING

Systemic signals are perceived in distant plant tissues and initiate systemic stress responses through priming (Gaupels and Corina Vlot, 2012). Knowledge on such long-distance signaling has been recently documented in various plant systems (Frost et al., 2008; Chaturvedi et al., 2012; Shah et al., 2014). According to the above studies, phloem is the likely path for systemic transmission or movement of signals associated with the acclimation process (Chaturvedi et al., 2012; Ruiz-Medrano et al., 2012). Similarly, root-applied NO or H_2O_2 remarkably increased NO and H_2O_2 steady-level in the leaves of citrus, indicating that these two molecules are systemic priming elicitors at the whole-plant level (Tanou et al., 2012a). Histochemical localization of H_2O_2 and $O_2^{\bullet-}$ production in citrus leaves using 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining indicated that ROS specifically accumulated in the vicinity of the primary vein (Tanou et al., 2012a). Such preferentially topological distribution of ROS reflects the greater exposure of periveinal cells to systemic signals, and that, when diffusing out of the veins, the concentration is diluted and therefore the cells near the vascular bundles are more likely to react (Alvarez et al., 1998). Further evidence for the long-distance and long-lasting nature of NO and H_2O_2 arose from the observation that both NO and H_2O_2-derived 4,5-diaminofluorescin diacetate (DAF-2DA) and 2,′7′-dichlorofluorescin diacetate (DCF-DA) fluorescence were detected in the vascular tissues (xylem and particularly phloem) and in the upper and lower epidermal cells under normal and NaCl stress conditions and occurred following 8 days of NO/H_2O_2 application (Tanou et al., 2012a). In citrus we also observed that the local application of NO in roots induced drought acclimation in leaves for at least 35 days after exposure to PEG stress (Ziogas et al., 2015) further suggesting that NO priming signal is memorized and systemically

transduced. However, the evidence for *in planta* NO and H_2O_2 long-lasting signaling is not strong enough to conclude their priming roles due to the short half-lives of NO and H_2O_2 (Neill et al., 2002). One mode of long-distance/lasting NO and H_2O_2 action may be associated with the auto-propagation of ROS and RNS waves throughout the plant (Mittler et al., 2011; Molassiotis and Fotopoulos, 2011). The initial abiotic stress-induced burst of ROS/RNS in a local group of plant cells triggers cell to cell communication that propagates throughout different tissues of the plant and carries a systemic signal over long distances (Miller et al., 2009). Another scenario might be the systemic NO and H_2O_2 signaling through binding to specific stress-related enzymes, such as mitogen-activated protein kinases (MAPKs) (Mishra et al., 2006) and *S*-nitrosoglutathione reductase (GSNOR) (Leterrier et al., 2011), thereby modifying their activity. In this regard, GSNOR transcripts in leaves and roots of salt-primed citrus plants were down-regulated showing that GSNOR could be considered a mechanism by which NO and H_2O_2 orchestrate priming signaling (Tanou et al., 2012a).

PROTEIN REPROGRAMMING IS A KEY MECHANISM OF NO AND H_2O_2-INDUCED PRIMING

Changes in environmental conditions are likely to cause rapid changes in the level, composition, and structure of different metabolites, proteins, and RNA molecules that precede signal transduction or stress acclimation events in plants (Baxter et al., 2014). An interesting finding that emerged from the work in citrus is the fact that the NaCl-responsive leaf proteome (85 proteins) was remarkably affected by pre-exposure to NO or to H_2O_2. Indeed, NO or H_2O_2 pretreatment prior to salt stress imposition, reversed a large part of the NaCl-responsive proteins (53 and 55 proteins, respectively; Tanou et al., 2009a). The major set of these proteins (46.7%) participate in photosynthesis and particularly in the Calvin cycle (e.g., several isoforms of Rubisco activase, Rubisco large subunit, fructose 1,6-bisphosphate aldolase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoribulokinase, transketolase, and carbonic anhydrase; Tanou et al., 2012a), this being a probable attempt at sustaining the photosynthesis rate during salt stress. Regulatory mechanisms of photosynthetic proteins were also studied in other stress-affected plant species and crops. For example, Komatsu et al. (2014) pointed the major proteomic alternation in wheat under various abiotic stresses focused on photosynthesis-responsive proteins. In addition to NO- or to H_2O_2-mediated regulation of protein expression during salinity acclimation, a more recent study showed that pre-treatment with NO also modulates specifically several PEG-affected proteins (e.g., glycolate oxidase, NADP-isocitrate dehydrogenase, and UPF0603 protein Atlg54780) in citrus plants experiencing priming against drought (Ziogas et al., 2015). These findings indicate that the priming function of NO and H_2O_2 in citrus plants is a dynamic, photosynthetic activity-demanding process that might be, at least partially, attributed to proteome reprogramming.

NO AND H_2O_2 -ALTERED PROTEIN POSTTRANSLATIONAL MODIFICATION PHYSIOGNOMY

It has been well documented that ROS and RNS exposure can prime plants against abiotic stresses through chemical reactions with specific target proteins that result in covalent posttranslational protein modifications (PTMs), altering protein function and activity (Lounifi et al., 2013). Therefore, the characterization of PTMs is crucial for a deeper understanding of NO and H_2O_2 priming. Particularly, protein carbonylation is a type of protein oxidation driven by oxidative stress, which occurs by direct attack on Lys, Arg, Pro, or Thr protein residues (Oracz et al., 2007). It has been shown that basal levels of protein carbonylation under control conditions increase sharply when citrus plants are grown under salinity (Tanou et al., 2009a, 2012a), inhibiting enzyme activities and increasing protein susceptibility toward proteolytic attack (Dunlop et al., 2002). Proteomic 2DE-OxyBlot-based analysis in citrus indicated that H_2O_2 and SNP pretreatments before salt stress prevented the NaCl-induced protein carbonylation to the levels of untreated control plants and allowed identifying 40 carbonylated proteins showing a reversal in accumulation level upon H_2O_2 or SNP application (Tanou et al., 2009a). Furthermore, tyrosine (Tyr) nitration, i.e., the addition of a nitro group (NO_2) to one of the two equivalent ortho carbons of the aromatic ring of Tyr residues in the presence of excess levels of ROS and NO or NO-derived species, is recognized as an important redox PTM (Corpas et al., 2009). Protein Tyr nitration has been established as a biomarker of systemic "nitroxidative stress," leading plant metabolism to a pro-oxidant status that disrupts NO signaling and induces protein structural and functional changes, some of which contribute to altered cell and tissue homeostasis (Corpas et al., 2013). Similar to the pattern of protein carbonylation, Tyr-nitration increased in citrus leaves exposed to salinity or to NO or H_2O_2 under stress-free conditions but diminished to control basal levels when these chemical treatments were applied before the imposition of NaCl stress (Tanou et al., 2012a). These results clearly show that citrus plants adopt a common oxy- and nitro-based stress-alleviating mechanism in their leaves. In both cases the results strengthen the notion that these PTMs are not just a 'fingerprint' of oxidative and nitrosative stress, but also they are essential components of citrus priming mechanism.

S-nitrosylation is another PTM that has been validated as signaling mechanism mediated by nitrosative/oxidative stress that occurs on cysteine (Cys) residues, being redox reversible with high spatial and temporal specificity modifying protein activity and accumulation (Astier and Lindermayr, 2012). In citrus leaves subjected to NaCl, *S*-nitrosylation decreased whereas NO or H_2O_2 pre-treatments before salt stress substantially increased protein *S*-nitrosylation. Remarkably,

this response was in contrast to protein carbonylation and Tyr-nitration patterns under the same experimental conditions (Tanou et al., 2009a, 2012a), denoting differences of PTMs regulation during salinity acclimation. By studying the ROS and RNS priming input against salinity we performed a comparative analysis of carbonylated, nitrated and nitrosylated proteome in citrus plants (Tanou et al., 2012a). This approach revealed that the majority of the PTM-targeted proteins in leaves were involved in Calvin–Benson cycle followed by disease/defense mechanisms and protein destination. More interestingly, among the 92 carbonylated, 88 Try-nitrated and 82 *S*-nitrosylated proteins, approximately one third of them, namely 34, 26 and 36 proteins respectively were specifically carbonylated or Try-nitrated or *S*-nitrosylated (Tanou et al., 2012a). On the contrary, 22 citrus proteins, including Rubisco large subunit, GAPDH subunits A, B, the photosystem II 44 kDa reaction center, sedoheptulose-1,7-bisphosphatase, phosphoribulokinase, carbonic anhydrase, light-harvesting chlorophyll a/b binding protein, ribulose-5-phosphate 3-epimerase, fructose-1,6-bisphosphate aldolase, glycolate oxidase and sinapyl alcohol dehydrogenase, simultaneously targeted by these three PTMs (Tanou et al., 2012a). It is also interesting that in a previous study we observed an overlap (17 proteins) among the NO– or H_2O_2–targeted carbonylated ($n = 40$) and *S*-nitrosylated ($n = 49$) proteins in citrus leaves experiencing priming effects against salinity (Tanou et al., 2009a), thereby disclosing that these proteins are common markers of NO and H_2O_2 signaling. Possible explanations for the co-modulated pattern of protein carbonylation and Tyr-nitration in contrast to *S*-nitrosylation have been proposed. The co-occurrence of more than one PTMs could be explained by the fact that the first PTM allosterically triggers the occurrence of other(s), thereby fine-tuning the degradation of damaged proteins by the proteasomes and/or offering protection against irreversible damage (Lounifi et al., 2013). It is therefore possible that *S*-nitrosylation might prevent the irreversible loss of function of proteins by protein oxidation due to carbonylation and/or Tyr-nitration by locking the structure of these proteins in a state under which they are no more sensitive to ROS/RNS attack (Sun et al., 2006). Altogether, the results support the existence of a link between oxidative and nitrosative regulatory events through PTMs characterizing priming phenomena in citrus plants.

LEAVES AND ROOTS EXHIBIT BOTH COMMON AND DISTINCT PRIMING CODES IN RESPONSE TO NO AND H_2O_2

When examining the expression of genes involved in NO production [e.g., nitric oxide synthase (NOS)-like proteins, nitrate reductase (NR), nitrite reductase (NiR)] as well as H_2O_2 generation [e.g., diamine oxidases (DAO) and polyamine oxidases (PAO)] we observed a distinctive complementary pattern in leaves and roots of citrus exposed to NaCl (Tanou et al., 2012a), likely reflecting locally and systemically differences in oxy/nitro priming signaling. Consistent with these tissue-specific transcriptional patterns, we also uncovered the existence of a tissue-dependent regulation of *S*-nitrosylation induced by NO and H_2O_2 stimuli (Tanou et al., 2012a). The *S*-nitrosylation was induced in leaves and depressed in roots following NO and H_2O_2 priming treatments, which is in agreement with the differential spatial distribution of oxidative and nitrosative stress in leaves versus roots documented in *Lotus japonicus* (Signorelli et al., 2013). By contrast, chemical pre-treatments with NO or H_2O_2 suppressed protein carbonylation and nitration in both leaves and roots exposed to NaCl, suggesting that some PTM responses are commonly regulated in the different types of citrus tissues (Tanou et al., 2012a). This is further strengthened by the fact that SNP stimulated Tyr-nitration in leaves and roots during acclimation to drought stress (Ziogas et al., 2015). Such functional differences between leaves and roots may be partially attributed to the tissue differences in the types of NO and ROS generation systems. Superoxide ($O_2^{\bullet -}$) and H_2O_2 produced through SODs up-regulation and polyamine degradation in roots of citrus just following pre-treatments with NO or H_2O_2 can activate specific signaling pathways distinct from those perceived by leaves (Tanou et al., 2012a). Analogously, NO accumulation via NR activation in citrus leaves subjected to NaCl or PEG stress (Tanou et al., 2012a) accompanies several different NO-signaling events which could regulate downstream pathways and stress acclimation (Baudouin and Hancock, 2014).

SIGNALING CROSS-TALK BETWEEN NO/ROS AND OTHER PATHWAYS

Recent studies have reported that many abiotic stress responses are coordinated by various signaling networks, particularly involving phytohormones, ROS, and RNS (Considine et al., 2015). In NaCl-treated citrus, NO and ROS accumulation in local and systemic tissues showed considerable overlap whereas a large part of the NaCl-sensitive proteins were commonly modulated by NO and H_2O_2 (Tanou et al., 2009a), suggesting that there is a dynamic interplay between the signals regulating priming. It is noted that comparative proteomic analysis in citrus leaves under physiological non-stressful conditions revealed (i) an interlinked NO- and H_2O_2-modulated protein network, (ii) the carbonylation status of a very large portion of the carbonylated citrus mitochondrial proteins, which remained constant or depressed by NO and H_2O_2 (Tanou et al., 2010), disclosing the parallels in action between NO and ROS. Here, we also provide examples demonstrating that NO could interact with other signaling pathways in modulating stress acclimation. For example, we observed temporal–spatial interactions between NO-specific PTMs and polyamines (PAs) homeostasis/metabolism in citrus challenged with salinity (Tanou et al., 2014), thus confirming that NO and PAs displayed some overlapping functions in plants (Parra-Lobato and Gomez-Jimenez, 2011). Targeted analysis of PA-affected *S*-nitrosylated citrus proteins led us to propose that PAs binding to specific

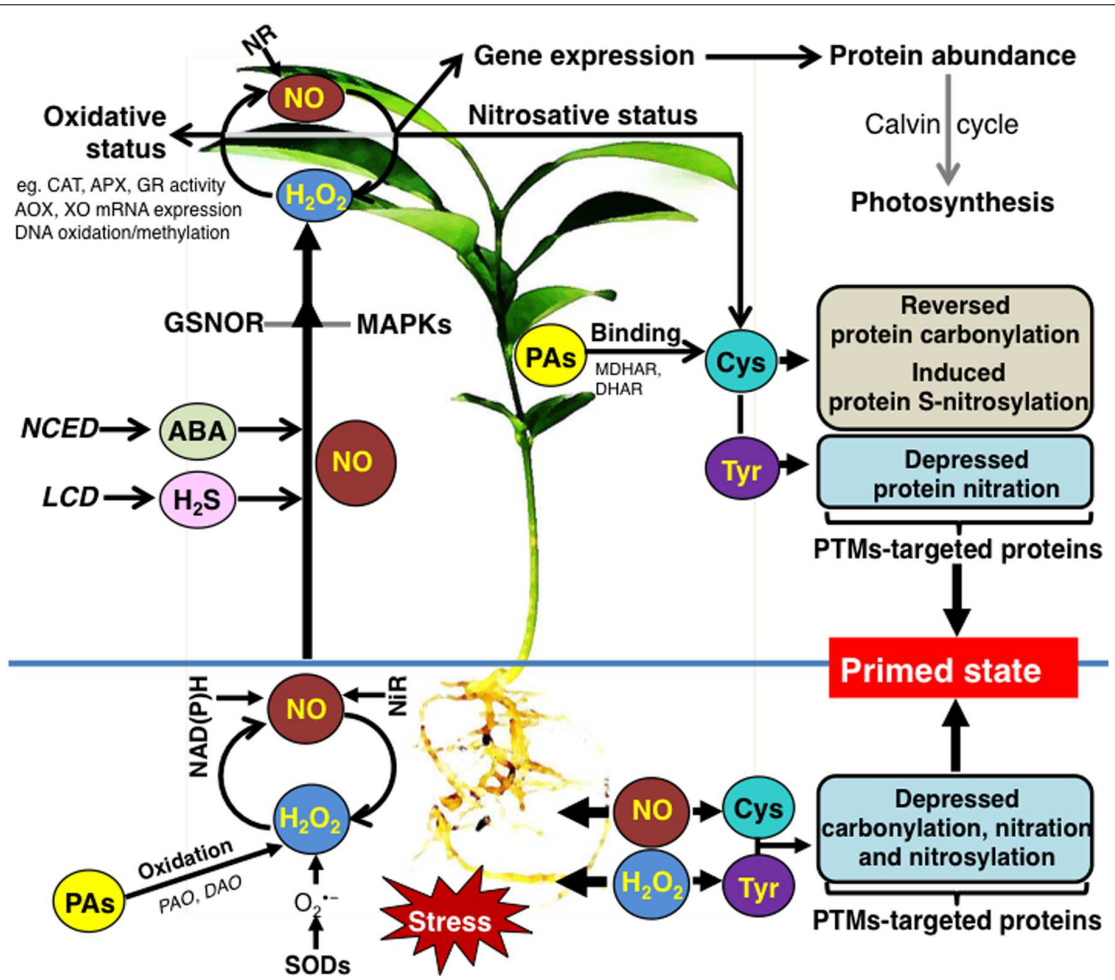

FIGURE 1 | Schematic overview of the signaling networks involved in the NO- and ROS-induced priming against salininy and drought stress in citrus plants (see text for details). ABA, abscisic acid; AOX, alternative oxidase; APX, ascorbate peroxidise; CAT, catalase; Cys, cysteine; DAO, diamine oxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSNOR, *S*-nitrosoglutathione reductase; H_2S, hydrogen sulfide; LCD, L-cysteine desulfhydrase; MAPKs, mitogen-activated protein kinases; MDHAR, monodehydroascorbate reductase; NAD(P)H, mitochondrial NAD(P)H dehydrogenases; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NiR, nitrite reductase; NR, nitrate reductase; PAO, polyamine oxidase; PTMs, posttranslational modifications; PAs, polyamines; SOD, superoxide dismutase; Tyr, tyrosine; XO, xanthine oxidoreductase.

proteins, such as dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR), may affect protein conformational parameters and also the environment surrounding Cys residues of protein targets (Tanou et al., 2014). Such chemical modifications of Cys residues could lead either to Cys oxidation through ROS or to *S*-nitrosylation through NO. Drought stress can induce accumulation of hydrogen sulfide (H_2S) and its biosynthetic enzyme L-cysteine desulfhydrase (LCD) in citrus. Moreover, in citrus roots H_2S can induce the expression of NiR and mitochondrial NAD(P)H dehydrogenases that are involved in NO production (Gupta et al., 2011) while H_2S accumulation acts downstream of NO in PEG-induced *S*-nitrosylation (Ziogas et al., 2015). Meanwhile, drought stress induction of ABA accumulation is a well-known fact (Tan et al., 2003). In citrus the fact that PEG stress-induced ABA accumulation and the expression of 9-*cis*-epoxycarotenoid dioxygenase (NCED), a key enzyme in ABA biosynthesis, were depressed in plants pre-exposed to NO or to H_2S (Ziogas et al., 2015) suggests that the interplay among NO, H_2S, and ABA could be considered a mechanism by which citrus orchestrates drought stress acclimation.

CONCLUSION AND FUTURE PERSPECTIVES

Research performed over the last years documented that NO and H_2O_2 induce priming toward salinity and drought in citrus plants and most importantly reveals key aspects of this phenomenon. Based on these results, we propose a signaling network through which NO and H_2O_2 provoke priming responses in leaves and roots of citrus (**Figure 1**). While several components of the priming mechanism have been proposed, we

still lack a thorough understanding of the complex mode of action of specific signaling molecules in plant stress acclimation. In this regard, various -omics techniques investigating both roots and leaves should be combined to fully understand NO and ROS-induced priming. It will be perhaps the major challenge of priming research to test these chemical agents against multiple abiotic stresses that occur in field conditions. Such an approach would allow establishing priming technology as a tool to manage crop yield.

AUTHOR CONTRIBUTIONS

AM, DJ, VZ, and GT wrote the paper.

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Reactive Nitrogen Species in Mitochondria and Their Implications in Plant Energy Status and Hypoxic Stress Tolerance

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Hypoxic and anoxic conditions result in the energy crisis that leads to cell damage. Since mitochondria are the primary organelles for energy production, the support of these organelles in a functional state is an important task during oxygen deprivation. Plant mitochondria adapted the strategy to survive under hypoxia by keeping electron transport operative even without oxygen via the use of nitrite as a terminal electrons acceptor. The process of nitrite reduction to nitric oxide (NO) in the mitochondrial electron transport chain recycles NADH and leads to a limited rate of ATP production. The produced ATP alongside with the ATP generated by fermentation supports the processes of transcription and translation required for hypoxic survival and recovery of plants. Non-symbiotic hemoglobins (called phytoglobins in plants) scavenge NO and thus contribute to regeneration of NAD^+ and nitrate required for the operation of anaerobic energy metabolism. This overall operation represents an important strategy of biochemical adaptation that results in the improvement of energy status and thereby in protection of plants in the conditions of hypoxic stress.

Keywords: peroxynitrite, nitric oxide, superoxide, hypoxia, mitochondria

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FUNCTIONALITY OF MITOCHONDRIA UNDER HYPOXIC STRESS

The primary function of mitochondria is to generate ATP, thus these organelles are vital for plant survival. Since oxygen is essential for ATP, any change in its concentration can affect ATP levels; therefore it can affect all energy requirements for biochemical reactions in the cell. Since mitochondria take up oxygen for respiration, these organelles efficiently sense oxygen. Plant tissues, especially roots, experience hypoxia during flooding and waterlogging (Bailey-Serres and Voesenek, 2008). Plants experience hypoxia in roots, germinating and developing seeds and in any bulky tissues due to the restricted diffusion of oxygen into these tissues through internal cell layers (Tschiersch et al., 2011). During hypoxia, cytochrome oxidase (COX) will have a limited capacity to function using oxygen while another terminal oxidase, the alternative oxidase (AOX), practically does not function under hypoxia (Igamberdiev and Hill, 2009). This is due to the different *K*m values of these two terminal oxidases. For instance, the *K*m value of COX for oxygen is in the range from 0.1 to 0.15 μM whereas the *K*m value for AOX is in the order of 10 μM (reviewed in Igamberdiev and Hill, 2009). The lack of the terminal acceptor alters the

mitochondrial functionality under hypoxia and near anoxia and results in impairment of the mitochondrial infrastructure (Vartapetian et al., 2003). The direct effect of oxygen deficiency on mitochondria is related to the lack of terminal electron acceptor in the electron transport chain and to the lack of ATP production. We will show below that plant mitochondria may have a sufficient metabolic plasticity to partially overcome these shortcomings, but their operation in such stress conditions is associated with the formation of reactive oxygen and nitrogen species.

PLANT MITOCHONDRIA APPLY VARIOUS STRATEGIES TO SURVIVE UNDER HYPOXIA

Survival under hypoxia depends on energy production. Therefore it is very important for the cells to safeguard mitochondria during stress. Plant mitochondria adapt various strategies to retain their structure for prolonged periods of time. One of such strategies is to keep the production of ATP under low oxygen conditions. A preliminary clue about the requisition for ATP for structural integrity came from study by Coueé et al. (1992), where the exogenous supply of ATP was shown to result in the support of endogenous ATP production and protection of mitochondria. Later several studies revealed that the application of nitrate has a protective role for root mitochondria from maize and pea seedling (Müller et al., 1994; Vartapetian and Polyakova, 1999). These authors suggested that nitrate (NO_3^-) can act as a terminal electron acceptor that supports the operation of electron transport chain in the absence of oxygen. But so far there is no evidence that nitrate can act as a terminal electron acceptor. How nitrate can be transported to mitochondria is also not known. Plants take up nitrate by high and low-affinity transporters (encoded by the families of genes *NRT1* and *NRT2*) (Dechorgnat et al., 2011). After being taken up by roots, NO_3^- is reduced first to NO_2^- by the cytosolic nitrate reductase (cNR) where NAD(P)H is used as electron donor, and further the plastidal nitrite reductase reduces nitrite to ammonium (Simontacchi et al., 2015). But there are no reports that mitochondria contain any nitrate transporter. On the other hand, nitrate could indirectly support the functionality of seedling mitochondria under hypoxia via its reduction to nitrite, which can be imported to mitochondria by a similar transporter to that found in chloroplasts (Sugiura et al., 2007) or by the mitochondrial inner membrane anion channel (PIMAC) activated under low ATP conditions (Laus et al., 2008). In potato tuber mitochondria, 29 metabolite transporters have been identified (Salvato et al., 2014), while 58 members of the mitochondrial carrier protein family were described in *Arabidopsis* (Palmieri et al., 2011), and 50 members in rice (Taylor et al., 2010). Some of these transporters can potentially carry nitrite, however, further studies are needed to establish what particular carrier is used to import nitrite into mitochondria.

The mitochondria of some anoxia-tolerant plant species do not change their structure under anoxia. For instance the mitochondria of anoxia-tolerant plants *Echinochloa phyllopogon* and *E. crus-galli* retained their structure and metabolic activity during the prolonged exposure to anaerobic stress (Kennedy et al., 1987). In another study it was shown that the mitochondrial biogenesis did not alter in the anoxia-tolerant rice (Howell et al., 2007). All this suggests that plants can use a specific strategy to survive under hypoxia or anoxia by keeping their mitochondria in a functional state (Gupta and Igamberdiev, 2011; Igamberdiev et al., 2014; Shingaki-Wells et al., 2014).

MITOCHONDRIA CAN REDUCE NITRITE TO NO

Several lines of evidence suggest that mitochondria of different and maybe all species are capable of reducing nitrite to nitric oxide (NO). For instance, the mitochondria isolated from ciliate protists and Fusarium fungus, possess a capability to reduce nitrite to NO (Tielens et al., 2002). Kozlov et al. (1999) showed that rat liver mitochondria can produce NO using nitrite. The first evidence for this reaction in photosynthetic organisms came from the study of the green alga *Chlorella sorokiniana* where the addition of nitrite resulted in NO formation and could be blocked by the inhibitors of the mitochondrial electron transport chain (Tischner et al., 2004). Later it was shown that tobacco cell suspensions are able to reduce nitrite to NO and that the application of mitochondrial inhibitors suppressed this reaction (Planchet et al., 2005). Then Gupta et al. (2005) conducted a detailed study and found that nitrite reduction to NO takes place in root mitochondria from various species, such as pea, barley, *Arabidopsis* and tobacco, and determined the *K*m value for nitrite reduction to NO (175 μM). This allowed estimating nitrite concentration needed for NO production. Since under hypoxia nitrite reduction to ammonium is inhibited (Botrel et al., 1996), the accumulated nitrite can act as a substrate for NO formation. This reaction is highly sensitive to oxygen, which has a *K*i value of approximately 0.05% or 0.6 μM (Gupta and Igamberdiev, 2011). Gupta and Kaiser (2010) demonstrated that this process occurs in the membrane but not in the matrix of mitochondria. The complexes III and IV of the mitochondrial electron transport chain were shown to be the sites for NO production.

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$$

The complex III can produce NO via a leakage of electrons to nitrite in a similar way as superoxide is produced by one-electron reduction of oxygen at this site. The mechanisms of nitrite reduction to NO by COX are still under investigation and several models are available for the explanation of this mechanism (reviewed in Gupta and Igamberdiev, 2011). The availability of oxygen, nitrite and NO determines the redox state of the COX center that contains heme a_3 and copper B ($Fe_{a3}Cu_B$) that in turn depends on the redox state of cytochrome *c*. In the absence of oxygen, Fe^{2+} donates the electron for nitrite reduction to NO. But still the concrete details of this mechanism remain speculative.

Other sites of nitrite reduction to NO in mitochondria may include cytochrome *c* itself and other hemeproteins (Basu et al., 2008). The proteins other than hemeproteins may also be

involved in NO formation. The involvement of the AOX, which is a di-iron carboxylate protein, in NO production was suggested on the basis of the effect of its inhibitor, salicylhydroxamic acid (SHAM) on NO evolution from mitochondria (Planchet et al., 2005; Gupta and Kaiser, 2010). Some proteins of a similar iron structure are found to be effective in NO metabolism (Kurtz, 2007). However, NO production in alfalfa (*Medicago truncatula*) nodules was fully insensitive to AOX inhibitor propylgallate (Horchani et al., 2011). The effect of AOX inhibitors reported in several studies may be explained by their action on other proteins, including peroxidases and other hemeproteins (Brouwer et al., 1986).

NITRITE REDUCTION TO NO LEADS TO ATP GENERATION VIA PHYTOGLOBIN-NO CYCLE

Under anoxia mitochondria produce significant amounts of NO (Gupta et al., 2005; Planchet et al., 2005). One interesting puzzle is the physiological role of the mitochondrial NO production. Previously it was suggested that COX may play a role in membrane translocation of protons during nitrite reduction to NO (Castello et al., 2006). Isolated mitochondria of barley and rice were able oxidize the externally applied NADH and NADPH under anoxia when nitrite was applied, and this oxidation resulted in the detectable ATP formation (Stoimenova et al., 2007). The build-up of ATP during the oxidation of NADH and NADPH by anoxic mitochondria was sensitive to myxothiazol and KCN suggesting that the operation of the complexes III and IV is essential for this process. The anoxic production of ATP constituted only 3–5% of the aerobic ATP generation. However, the ATP produced during glycolytic fermentation together with the mitochondrial anaerobic nitrite-driven ATP production can make a major contribution for hypoxic survival.

The produced NO needs to be recycled very fast in order to avoid the nitrosative stress. Plants possess hypoxically induced hexacoordinated hemoglobins (called class 1 phytoglobins). They are the active scavengers of NO; thereby they can significantly reduce the amounts of NO to the levels that are much less toxic to plants. Class 1 phytoglobins possess the affinity to oxygen of two orders of magnitude higher than cytochrome *c* oxidase (*K*d ∼2 nM); therefore they can operate at the oxygen levels that cannot be utilized by mitochondria (Igamberdiev et al., 2011). Scavenging of NO involves the oxidation of phytoglobin ferrous ion, forming metphytoglobin and nitrate (Igamberdiev et al., 2006, 2011). Operation of the phytoglobin-NO cycle is essential for recycling nitrate and nitrite and for oxidation of excess of NADH and NADPH (Igamberdiev and Hill, 2004, 2009; Gupta and Igamberdiev, 2011; Igamberdiev et al., 2014) (**Figure 1**). This cycle can also contribute to NAD^+ recycling for the continuous operation of glycolysis. Its operation is important not only under the hypoxic conditions (that occur during germination of seeds, in flooding stress or in compact meristemic tissues) but also in the course of pathogen infection, when significant amounts of NO are formed during the response mediated by salicylic and jasmonic acids (Mur et al., 2012).

IS NITRITE-DRIVEN ANAEROBIC ATP SYNTHESIS SUFFICIENT FOR PLANTS TO SURVIVE UNDER HYPOXIA?

In order to survive under hypoxia and anoxia, plants need to sustain the machinery to carry out transcription and translation. It has been shown that the energy budgeting occurs under hypoxia to direct a specific amount of ATP for cellular functions (Edwards et al., 2012); at the same time cell recovery processes, such as cell division and elongation, decelerate in order to save energy (Takahashi et al., 2011). Several lines of evidence suggest that the active transcription of the mitochondrial genes takes place under hypoxia (Narsai and Whelan, 2013), while RNA translation appears to be downregulated. This was evidenced by 50% reduction in polysome content (Branco-Price et al., 2008) to reduce energy costs of unwanted translation during stress condition.

For instance, plants actively switch on the ethylene responsive transcription factors which are required for hypoxia sensing and survival (Bailey-Serres et al., 2012). In comparison to transcription, protein biosynthesis is much more energy demanding process. It has been shown that the translation of catabolic proteins takes place in anoxic coleoptiles of rice (Edwards et al., 2012). Plants invest energy in making catabolic proteins, such as the enzymes involved in glycolysis, under hypoxia/anoxia to get higher energy yield. Previously we have shown that mitochondria from roots produce NO within minutes of exposure to hypoxia suggesting that energy production initiation related to NO turnover starts within minutes. The anoxia-tolerant rice produced much higher levels of anoxic ATP in comparison to anoxia-intolerant barley suggesting essentiality of the nitrite-driven ATP production in the anoxic survival (Stoimenova et al., 2007). Since nitrate reductase (NR) is a part of the hemoglobin-NO cycle, its transcript is highly induced in anoxic rice coleoptiles (Lasanthi-Kudahettige et al., 2007). The increase of NR activity under oxygen deprivation (Planchet et al., 2005) suggests that the activation of transcription and translation of NR contributes to the excess of nitrite production and to NO generation, thus helping to improve the energy status of hypoxic plants to support the transcription and translation processes. The post-translational activation of NR under hypoxia is particularly important, which is achieved through dissociation of the 14-3-3 protein inhibitor and NR dephosphorylation (Allègre et al., 2004).

ROS IN THEIR REACTION WITH NO PRODUCE REACTIVE NITROGEN SPECIES (RNS)

Electrons enter the mitochondrial electron transport chain primarily via complex I, the alternative rotenone-insensitive dehydrogenases, and complex II (succinate dehydrogenase). These electrons are transferred to the ubiquinone pool which is a central reservoir for accumulation of electrons. From the ubiquinone pool electrons pass to complex III and complex IV,

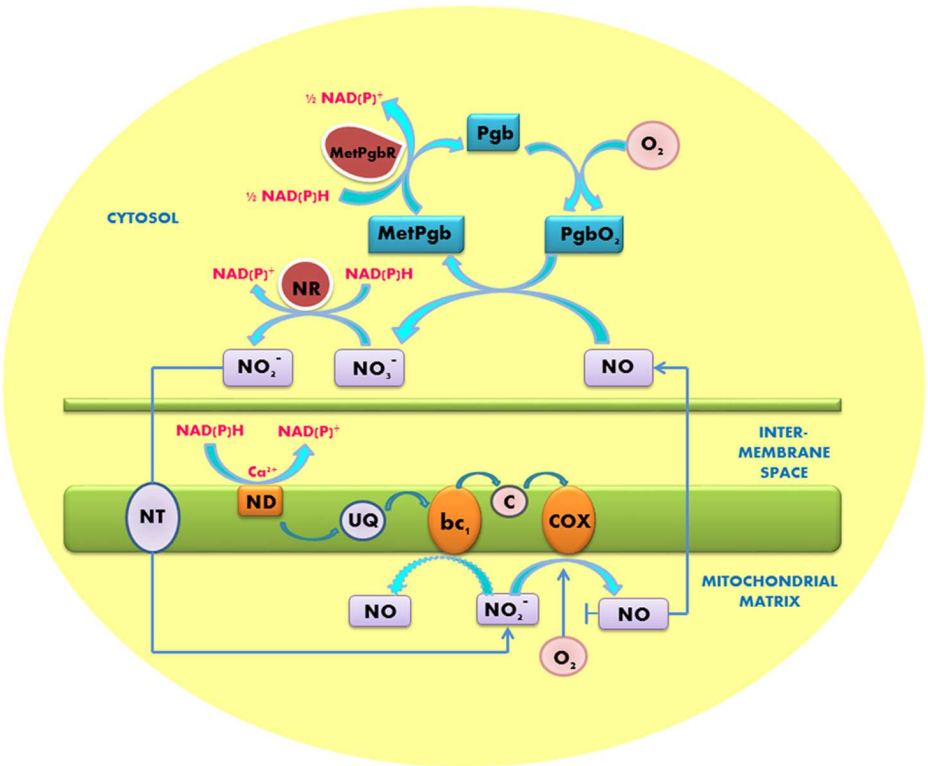

FIGURE 1 | Operation of the phytoglobin/NO cycle between the anoxic mitochondrion and cytosol. Nitrite reduction and NO formation occur at COX and complex III (bc_1). NO diffuses to the cytosol where it is converted to nitrate (NO_3^-) by the oxygenated non-symbiotic phytoglobin ($PgbO_2$). Metphytoglobin (MetPgb) formed in this reaction is recycled by metphytoglobin reductase (MetPgbR) and the product nitrate is reduced by nitrate reductase (NR) to nitrite, which is transported to mitochondria by a putative nitrite transporter (NT). NAD(P)H is oxidized by the externally facing mitochondrial dehydrogenases (ND). Q, ubiquinone; c, cytochrome *c*. Modified from Gupta and Igamberdiev (2011).

or to the AOX. The transfer through complexes III and IV leads to the generation of electrochemical proton gradient which is used by the ATP synthase to generate ATP (Rasmusson et al., 2008). When oxygen concentrations decrease below 10 μM, AOX does not function due its high *K*m to oxygen, and when oxygen concentrations fall further below 1 μM, the operation of COX is also ceased. This leads to the accumulation of electrons in the ubiquinone pool and at the other sites such as complex I and III. This results in the condition when the remaining oxygen molecules accept only one electron which leads to the production of superoxide anion (O_2^-). The elevated $NADH/NAD^+$ ratio in the mitochondrial matrix under hypoxic conditions, becomes a condition leading to the increase in superoxide anion production even at low oxygen concentrations (Murphy, 2009). The reduced electron carrier proteins are able to react with O_2 to form O_2^-, while NO produced under hypoxia can activate or repress these proteins to control O_2^- production. NO is known to inhibit respiration by competitively binding to cytochrome c oxidase (Cooper, 2002). Thus, by increasing or decreasing the rate of O_2 consumption by mitochondria NO may influence O_2^- production *in vivo* by altering the local $[O_2]$ (Borisjuk et al., 2007). Recently it was shown that the overexpression of phytoglobins leads not only to the decrease in NO but also results in the increased respiration, lowering internal oxygen concentration, and subsequent production of ROS (Gupta et al., 2014). Paradoxically, the mitochondrial O_2^- increases in response to low oxygen levels (Chandel et al., 1998; Guzy and Schumacker, 2006).

The produced superoxide is responsible for the generation of other ROS and of RNS. For instance, superoxide dismutase converts superoxide to hydrogen peroxide which can act as a signal. Its excessive amounts lead to cytotoxicity. NO does not directly react with H_2O_2 but there are reports that NO can reduce H_2O_2 formation (Małolepsza and Rózalska, 2005). This can be explained, in particular, by the activation through *S*-nitrosylation of ascorbate peroxidase, which is the key enzyme participating in H_2O_2 scavenging (Correa-Aragunde et al., 2013; Begara-Morales et al., 2014; Yang et al., 2015). The reaction between superoxide and NO occurs near the diffusion controlled rates and results in the formation of peroxynitrite ($ONOO^-$), which is a toxic RNS form causing tyrosine nitration (Poyton et al., 2009). Peroxynitrite is formed in relatively low amounts under non-stress conditions, while under stress both NO and superoxide levels increase stimulating its formation. The biological reactions of NO and superoxide that limit their availability also constrain the amount of peroxynitrite formed. SOD competes effectively with NO for superoxide by reducing its level and decreasing the amount of peroxynitrite formed. The class 1 phytoglobin also

decreases peroxynitrite concentration by reducing the availability of NO to react with superoxide. Peroxynitrite can be scavenged in plants via the pathway involving thioredoxin (Wulff et al., 2009), which results in its lower toxicity in plants as compared to animal tissues where its scavenging is likely limited by a side reaction of cytochrome *c* oxidase (Pearce et al., 2002). The excess of NO under hypoxia can react with peroxynitrite resulting in the formation of non-toxic nitrogen dioxide (NO_2) and nitrite (NO_2^-). The produced NO_2 can react with NO which then leads to the formation of dinitrogen trioxide (N_2O_3) which plays a role in nitrosative reactions (Espey et al., 2002).

In summary, plant mitochondria in the conditions of oxygen deficiency can reduce nitrite to NO, which can help in increasing their energy efficiency for supporting active transcription and translation processes in the hypoxic cells. NO participates in NAD^+ recycling via the hemoglobin-NO cycle. In the reactions with ROS, NO forms peroxynitrite and other RNS such as N_2O_3 which play a role as signals during the nitrosative stress.

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frontiers in Plant Science

ORIGINAL RESEARCH
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Canavanine Alters ROS/RNS Level and Leads to Post-translational Modification of Proteins in Roots of Tomato Seedlings

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Canavanine (CAN), a structural analog of arginine (Arg), is used as a selective inhibitor of inducible NOS in mammals. CAN is incorporated into proteins' structure in the place of Arg, leading to the formation of aberrant compounds. This non-protein amino acid is found in legumes, e.g., *Canavalia ensiformis* (L.) DC. or *Sutherlandia frutescens* (L.) R.Br. and acts as a strong toxin against herbivores or plants. Tomato (*Solanum lycopersicum* L.) seedlings were treated for 24–72 h with CAN (10 or 50 μM) inhibiting root growth by 50 or 100%, without lethal effect. We determined ROS level/production in root extracts, fluorescence of DAF-FM and APF derivatives corresponding to RNS level in roots of tomato seedlings and linked CAN-induced restriction of root growth to the post-translational modifications (PTMs) of proteins: carbonylation and nitration. Both PTMs are stable markers of nitro-oxidative stress, regarded as the plant's secondary response to phytotoxins. CAN enhanced H_2O_2 content and superoxide radicals generation in extracts of tomato roots and stimulated formation of protein carbonyl groups. An elevated level of carbonylated proteins was characteristic for the plants after 72 h of the culture, mainly for the roots exposed to 10 μM CAN. The proteolytic activity was stimulated by tested non-protein amino acid. CAN treatment led to decline of fluorescence of DAF-FM derivatives, and transiently stimulated fluorescence of APF derivatives. Short-term exposure of tomato seedlings to CAN lowered the protein nitration level. Activity of peroxidase, polyamine oxidase and NADPH oxidase, enzymes acting as modulators of H_2O_2 concentration and governing root architecture and growth were determined. Activities of all enzymes were stimulated by CAN, but no strict CAN concentration dependence was observed. We conclude, that although CAN treatment led to a decline in the nitric oxide level, PTMs observed in roots of plants exposed to CAN are linked rather to the formation of carbonyl groups than to nitration, and are detected particularly after 24 h. Thus, oxidative stress and oxidative modifications of proteins seems to be of significant importance in the rapid response of plants to CAN.

Keywords: NADPH oxidase, peroxidase, polyamine oxidase, proteolysis, protein carbonylation, protein nitration, RNS, ROS

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INTRODUCTION

In addition to 20 amino acids, known to act as building blocks of proteins, living organisms also produce non-proteinogenic amino acids (NPAAs). In plants, about 250 NPAAs have been identified (Vranova et al., 2011), which possess a rich structural diversity and some of them exhibit harmful biological effects both in plants and animals (Rosenthal, 1982, 2001; Bell, 2003). The possible role of NPAAs in plants is protection against predators or pathogens. They act also as an allelopathic weapon against neighboring organisms. Among NPAAs naturally occurring in planta L-canavanine (CAN), the L-2-amino-4-(guanidinoxy) butyric acid is identified and recognized as a compound of high toxicity (Rosenthal, 2001), but of great importance in medicine, where is used as cytotoxic agent against cancer cells in humans (Swaffar et al., 1994, 1995; Vynnytska et al., 2011). In animals the mode of action of CAN depends on the fact that this NPAA is the guanidinoxy structural analog of arginine (Arg) and its presence can lead to a production of CAN-containing proteins, which may disrupt cellular metabolism (Rosenthal and Harper, 1996). CAN affects also regulatory and catalytic reactions of Arg metabolism or uptake. According to the toxicological data, CAN is considered to be "very toxic" (Rodricks, 2007), as is linked with several serious diseases in humans, e.g., systemic lupus erythematosus (SLE), which is characterized by a defect in the immune system. The inflammatory and oxidative modification reactions are the most important events associated with complications of SLE patients (Shah et al., 2014). Recently, hyper-nitration of tyrosine residues of, e.g., histone H1 has been suggested as an etiopathogenesis of SLE and rheumatoid arthritis (Khan et al., 2014).

In mammalian tissue NO synthase (NOS) converts Arg to NO and L-citrulline (Stuehr, 1999). Acting as an antimetabolite of Arg, in animal tissue CAN is commonly used as a selective inhibitor of inducible isoform of NOS (iNOS; Abd El-Gawad and Khalifa, 2001; Li et al., 2001). Application of CAN in animal or human tissue to inhibit NOS activity results in a decrease in NO emission (Luzzi and Marletta, 2005), but also induces an oxidative burst demonstrated by an elevated level of ROS (Demiryürek et al., 1997; Riganti et al., 2003).

In plants, like in animal tissue, NO acts as a signaling molecule. But the pathways of its biosynthesis are still not clarified. Among many metabolic pathways such as polyamines (PAs) or ethylene biosynthesis, in plant cells Arg is the hypothetical substrate for NO formation. There is no doubt that in higher plants NO formation depends on reductive pathways utilizing NO_2^- as a substrate, but the existence of the enzyme of NOS-like activity is controversial (Corpas et al., 2009; review by Gupta et al., 2011; Hancock, 2012; Corpas and Barroso, 2015). Nevertheless, both in animals and plants NO mode of action involves PTMs of proteins including *S*-nitrosylation of cysteine (Cys) and nitration of tyrosine (Tyr) residues (Lozano-Juste et al., 2011). In addition, carbonylation of bovine serum albumin (BSA) has been demonstrated *in vitro* after NO donor application (Krasuska et al., 2014b). Nitration of Tyr is connected with a covalent binding of a nitro (-NO_2) group to one of the two equivalent *ortho* carbons of this amino acid and leads to the formation of 3-nitrotyrosine (NO_2Tyr, 3-NT; Chaki et al., 2009). Nitration is associated with an accumulation of peroxynitrite ($ONOO^-$), the oxidizing product of reaction of superoxide anion ($O_2^{\bullet -}$) and NO in its radical form (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2011; Astier and Lindermayr, 2012).

Disturbances in the metabolism of NO or other RNS are related to an alteration in ROS level leading commonly to the induction of nitro-oxidative stress (Corpas and Barroso, 2013). The formation of carbonylated proteins is due to various types of oxidative modification of amino acids, direct ROS attacks on proline (Pro), Arg, lysine (Lys), asparagine (Asn) and threonine (Thr) residues, the incorporation of reactive carbonyl derivatives into peptides by interaction with Cys, histidine (His) and Lys or the adduction of advanced glycation end products formed by ROS reactions with reducing sugars (Møller et al., 2011; Yan and Forster, 2011). It has been proposed that carbonylation, although is a negative consequence of ROS over-accumulation, serves also as an intracellular metabolic regulatory mechanism.

Treatment of tomato (*Solanum lycopersicum* L.) seedlings with CAN (10 μM) for 24 or 72 h led to 50% inhibition of root growth, while the application of CAN at higher concentration (50 μM) entirely inhibited root elongation, but had no lethal effect, although no recovery was observed after seedlings transfer to Arg or water (Krasuska et al., 2016). CAN treatment did not lead to DNA fragmentation, but resulted in slight enhancement in RNA content, accompanied by 20% enlargement in total protein level. Preliminary data indicated that prolonged culture of tomato seedlings in CAN solution increased accumulation of ROS ($O_2^{\bullet -}$ and H_2O_2) in tomato root tips detected by tissue staining with NBT or DAB (Krasuska et al., 2016). CAN inhibited also NO emission and transiently enhanced $ONOO^-$ production in root tips of tomato seedlings, suggesting its direct impact on NO biosynthetic pathway (Krasuska et al., 2016).

The aim of our work was to show another than incorporation into the protein structure mode of action of CAN. We investigated the modifications in ROS concentration or generation rate, and fluorescence of DAF-FM or APF derivatives after reaction with RNS in roots of tomato seedlings after 24–72 h long exposure to CAN, as our previously published data indicated influence of CAN on RNS content in root tips (Krasuska et al., 2016). Following the treatments, formation of carbonyl groups and 3-NT were studied, to detect stable and reliable markers of nitro-oxidative stress, which could be induced by CAN. Some PTMs, e.g., carbonylation stimulate proteolytic degradation, therefore, we investigated impact of CAN on proteolytic activity. In addition, we linked CAN toxicity with the activity of polyamine oxidase (PAO) because decline of PAs content was observed as a result of CAN treatment (Davis, 1997) and degradation of PAs leads to H_2O_2 formation. NADPH oxidase catalyze the apoplastic production of $O_2^{\bullet -}$, plant

Abbreviations: Arg, arginine; CAN, canavanine; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; 3-NT, 3-nitrotyrosine; NO, nitric oxide; PTMs, post-translational protein modifications; RNS, reactive nitrogen species; ROS, reactive oxygen species.

peroxidase Class III (POx) catalyzes oxidoreduction between H_2O_2 and various reductants (Hiraga et al., 2001; Kaur et al., 2014). Both enzymes govern also root architecture and growth, together with classical phytohormons and NO (Krasuska and Gniazdowska, 2015) therefore, could play an important role in plant response to CAN.

To summarize, the aim of the study was to find the stable markers of CAN toxicity and describe the mode of action of this non-protein amino acid, to show that application of commonly used inhibitor of NO biosynthesis may result in oxidative modifications.

MATERIALS AND METHODS

Plant Material

Seeds of tomato (*Solanum lycopersicum* L. cv. Malinowy Ożarowski) were germinated in water at 20°C in darkness for 3 days. After this period seedlings of equal roots' length (5 mm) were separated and transferred to Petri dishes (Ø15 cm) containing filter paper moistened with distilled water (control) or CAN (Sigma-Aldrich) dissolved in distilled water pH 7.5 (**Figure 1**). Control seedlings and seedlings treated with CAN were cultured in a growth chamber at 23/20°C, 12/12 h day/night regime for 24 or 72 h.

The concentration of CAN (10 μM) required for reduction of root length to 50% of the control, was accepted as IC_{50}. The concentration (50 μM), which completely inhibited elongation growth of roots, was accepted as IC_{100}.

Measurement of Hydrogen Peroxide (H_2O_2) Concentration

The concentration of H_2O_2 in extracts of tomato roots was determined according to Velikova et al. (2000). Isolated roots (100 mg) were immediately homogenized by pestle and mortar in cooled 0.1% (w/v) trichloroacetic acid (TCA). After centrifugation at 15,000 *g* for 15 min at 4°C, the supernatant was collected for further analysis. The concentration of H_2O_2 was measured in the assay mixture (0.25 ml supernatant, 1 ml freshly prepared 1 M KI in 10 mM potassium phosphate buffer pH 7.0, and 0.5 ml 10 mM potassium phosphate buffer pH 7.0) at 390 nm using a Hitachi U-2900 spectrophotometer. The standard curve was prepared using 0.88 μM H_2O_2 (Sigma-Aldrich). Measurements of H_2O_2 concentration were done in three independent experiments, each in three biological replicates and expressed as nmol g^{-1} FW.

Using the same method, the H_2O_2 concentration was measured in the liquid medium surrounding the roots. An equal number of control or CAN-treated tomato seedlings were placed in Petri dishes (Ø7.5 cm), and the culture was carried out as was described in plant material section. Then, the whole solution from the plate of the culture was collected, the volume was measured, and 0.5 ml was taken for the analyses in the reaction mixture. H_2O_2 concentration in the culture solution was measured at 390 nm. As a blank, water or CAN solutions from the plates without plant material were used. The experiment was done in four repetitions, and expressed as nmol L^{-1}.

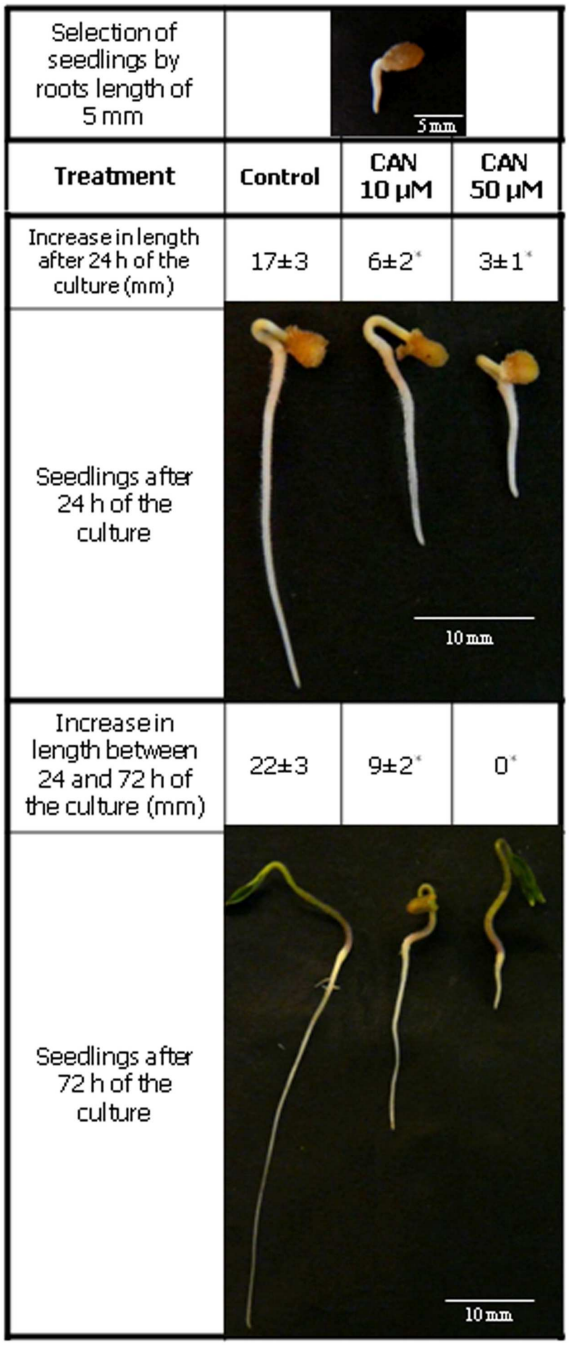

FIGURE 1 | Tomato seedlings morphology. Equal of size, germinated seeds of tomato, with roots of 5 mm long were grown in water (control) and 10 or 50 μM CAN solution for 72 h. Increase in length of root was measured twice during the culture period: 24 h after seedlings transfer to CAN and then after additional 48 h of experiment. Values are average ± SE of five independent experiments. Asterisk (*) indicate significance from control at the same time of culture period at $p \leq 0.05$ based on Student's test.

Measurement of Oxidation of Epinephrine by Superoxide Radicals in Root Extract

Measurement of oxidation of epinephrine by superoxide radicals was done according to Misra and Fridovich (1972). Roots of tomato seedlings (60 mg) were homogenized in 0.05 M Tris-HCl pH 7.5 with addition of 2% (w/v) polyvinylpolypyrrolidone (PVPP), and centrifuged at 12,000 *g* for 15 min at 4°C. The supernatant was immediately used for further analyses. The oxidation of epinephrine to adrenochrome was measured in reaction mixture (0.05 ml of supernatant, 0.05 ml 0.05 M Tris-HCl pH 7.5, 0.05 ml of 60 mM epinephrine prepared in 50 mM HCl) at 480 nm (microplate reader Sunrise, Tecan) for 2 min. Autooxidation of epinephrine in the reaction mixture (without roots extract) was done in each assay and obtained values were included in the calculations. The epinephrine extinction coefficient was $\varepsilon = 4.02\ mM^{-1}\ cm^{-1}$. The measurements were done in three independent experiments, each in three biological replicates and expressed as relative units. One relative unit corresponds to the rate of epinephrine oxidation in root extracts of control tomato seedlings after 24 h of culture calculated as $\mu mol\ min^{-1}\ g^{-1}$ FW.

Measurement of Fluorescence Emission Corresponding to NO and $ONOO^-$ Level in Tomato Roots

Nitric oxide and $ONOO^-$ generation was measured as efflux of derivatives of 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA, Invitrogen) and 3′-(*p*-aminophenyl) fluorescein (APF, Invitrogen), respectively following the manufactere's instructions. APF is sensitive to $ONOO^-$ but also to $OH^{\bullet}$, and hypochlorite (Halliwell and Whiteman, 2004).

For NO analyses, roots of three plants were washed twice in distilled water and incubated for 40 min in darkness at room temperature in the mixture: 20 μM DAF-FM DA [2 mM DAF-FM DA prepared as stock solution dissolved in dimethyl sulfoxide (DMSO)] in 10 mM HEPES-KOH, pH 7.4. After incubation, roots were washed twice in 10 mM HEPES-KOH pH 7.4 and transferred to a cuvette containing 2 ml of this buffer. Fluorescence was recorded for 1,500 s (excitation 495 nm, emission 515 nm) using a Hitachi F-2500 spectrofluorimeter. A final intensity of fluorescence in the buffer above the roots, after the indicated time was taken into calculations. After measurement the roots were gently dried on filter paper and weighed.

For $ONOO^-$ determination, two roots were washed twice in distilled water and then incubated for 1 h in darkness at room temperature with 0.2% (v/v) APF [5 mM APF – stock solution dissolved in dimethylformamide (DMF)] in 10 mM HEPES-KOH, pH 7.2. After incubation roots were washed twice in 10 mM HEPES-KOH, pH 7.2 and transferred to a cuvette containing 2 ml of this buffer. Fluorescence was recorded for 1,800 s (excitation 490 nm, emission 515 nm) using a Hitachi F-2500 spectrofluorimeter. A final intensity of fluorescence in the buffer above the roots, after the indicated time, was taken into calculations. After measurement the roots were gently dried on filter paper and weighed.

Fluorescence was calculated per 1 g FW and expressed in arbitrary units (U). 1 U was estimated from the result obtained for roots isolated before treatment with CAN.

Measurements were done in 3–4 independent experiments with three repetitions in each.

Quantitative Measurement of Protein Carbonyl Groups

Protein carbonyl groups in root extracts were measured using enzyme-linked immunosorbent assay (ELISA) according to Levine et al. (1994) and Buss et al. (1997). Roots (200 mg) were homogenized in 0.1 M Tris-HCl, pH 7.0 with 1 mM ethylenediaminetetraacetic acid (EDTA), 2% (w/v) PVPP, 1 mM dithiothreitol (DTT), 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich P9599), and 10% (v/v) glycerol in an ice bath. After centrifugation at 15,000 *g* 15 min at 4°C, the supernatant was collected and incubated with 1% (w/v) streptomycin (for 20 min, at room temperature, in darkness with constant slow shaking). Then, supernatant was incubated with 10 mM 2,4-dinitrophenylhydrazine (DNPH; Sigma-Aldrich) dissolved in DMSO in the dark for 35 min at 37°C. At the same time, samples prepared without DNPH were incubated in 2 M HCl. Proteins were precipitated 10 min with 10% (w/v) TCA, and the pellets obtained after centrifugation (10,000 *g*; 10 min) were washed three times with 1:1 (v/v) ethanol:ethyl acetate. After each washing step, the samples were centrifuged for 5 min at 10,000 *g*. Washed pellets were dissolved in 6 M guanidine hydrochloride (Sigma) in 2 M HCl. Protein concentration was measured according to Bradford (1976) using BSA as a standard. Reduction of BSA fatty acid free (Sigma-Aldrich) was done using sodium dithionite (2 mM) for 30 min at 25°C in darkness. Solution of reduced BSA was passed through Sephadex G-25 column equilibrated with 0.1 M Tris-HCl, pH 7.0. Oxidation of reduced BSA was done using 1 μl of 3% (v/v) H_2O_2 with the addition of sodium perchlorate for 30 min at room temperature in darkness. Carbonyl groups in oxidized BSA were labeled with DNPH and processed as described above. Blank probes of BSA were incubated in 2 M HCl without addition of DNPH. Final BSA concentration was determined using Bradford reagent. Concentration of carbonyl groups in BSA was measured at 375 nm, and calculated from the extinction coefficient $\varepsilon = 22\ mM^{-1}\ cm^{-1}$.

Before measurement, the pH of the protein samples and oxidized BSA was adjusted to 9.0 with 5 M KOH. Triplicate of 200 μl of each sample or oxidized BSA were added to wells of Nunc Immuno Plate Maxisorp (Sigma), and incubated at 4°C overnight. Next day the samples were removed and plates were washed three times with Tris-buffered saline (TBS) with 0.05% (v/v) Tween-20 (TBST). Next, the plate wells were blocked with 0.1% (w/v) BSA in TBS (250 μl per well) for 1.5 h at room temperature. After washing step (three times with TBST), monoclonal primary antibodies (Monoclonal Anti-dinitrophenyl (DNP) antibodies, A2831 Sigma-Aldrich)

conjugated with alkaline phosphatase (dilution 1:25,000) in TBST were added, at room temperature for 1.5 h in darkness. After removal of antibodies and another washing step (three times with TBST) *p*-nitrophenylphosphate disodium (pNPP), prepared at concentration 1 mg ml^{-1} in 1 M diethanolamine (DEA) solution with 0.5 mM $MgCl_2$ was added into each well. Incubation of the plates were carried out at 37°C for 1 h, the reaction was stopped with 5 μl of 5 M KOH, and absorbance was read at 405 nm with referential wave 605 nm in microplate reader (Sunrise, Tecan). Measurement of protein carbonyl groups were done in three biological repetitions, each in three replicates and expressed as nmol mg^{-1} protein.

Immunodetection of Carbonyl Groups

Immunodetection of protein carbonyl groups was done by immunoblotting technique. Protein samples prepared as described above were suspended in the sample buffer: 63 mM Tris-HCl, pH 6.8, 1% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, and 20 mM DTT. After incubation at 95°C for 10 min, 20 μg proteins were loaded per lane, and separated on 10% polyacrylamide gels with SDS (SDS-PAGE) according to Laemmli (1970), and then electrotransferred to nitrocellulose membranes (Pure Nitrocellulose Membrane, Sigma-Aldrich) according to Towbin et al. (1979) using a Bio-Rad wet blotting apparatus. Subsequently, the proteins were visualized on the membranes by incubation in 0.2% Ponceau S dissolved in 2% acetic acid. The membranes were blocked overnight at 4°C with non-fat dry milk in TBST. After blocking, membranes were washed three times in TBST, and immunolabeling of carbonyl groups was carried out by incubating the membranes with monoclonal anti-DNP antibodies, conjugated with alkaline phosphatase at a dilution of 1:100,000 at room temperature. Visualization of carbonylated proteins was done after addition of 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$, 0.2 mM nitroblue tetrazolium (NBT), and 0.21 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Assays were performed in 2–3 independent experiments and typical results are shown. The entire gel lanes were quantified by densitometry analysis, which was done using Image J.

Quantitative Measurement of 3-Nitrotyrosine in Proteins

3-NT modified proteins were analyzed by an ELISA method according to Khan et al. (1998). Roots of tomato seedling (200 mg) were washed twice in distilled water and homogenized by mortar and pestle in 0.1 M HEPES-KOH, pH 7.0 with 1 mM EDTA, 2% (w/v) PVPP, 2 mM DTT, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich) and 10% (v/v) glycerol in an ice bath. Following centrifugation at 15,000 *g* for 15 min, 4°C, the supernatant was collected for protein determination.

Positive control was prepared from fatty acid free BSA dissolved in TBS. BSA was incubated with $NaNO_2$ (1 mM) acidified with 0.2 M HCl, and in the presence of 0.1 mM $NaHCO_3$ for 30 min at 37°C, in darkness. After incubation, BSA was precipitated with 20% (w/v) TCA for 20 min at room temperature, centrifuged (10,000 *g*; 10 min), and dissolved in water adjusted to pH 9.0 with 1 M KOH. The 3-NT content of nitro-BSA was determined at 438 nm, and calculated from the extinction coefficient $\varepsilon = 4.3\ mM^{-1}\ cm^{-1}$.

Root protein samples before ELISA measurement were adjusted to pH 9.0 with 1 M KOH. Nunc Immuno Plate Maxisorp (Sigma) was coated with samples or nitrated-BSA (200 μl per well), and incubated at 4°C overnight. After incubation, the plate was washed three times with PBS. Following blocking step with 0.1% (w/v) gelatin in TBS (250 μl per well; 1.5 h at 37°C in darkness) and washing three times with TBST, monoclonal primary antibodies (Monoclonal Anti-3-Nitrotyrosine antibodies, Sigma-Aldrich) were added at a dilution of 1:1,000 (200 μl per well), and incubated for 1 h at 37°C in darkness. After washing three times with TBST, the plate was covered with secondary antibodies (anti-mouse IgG conjugated with Alcaline Phosphatase Sigma-Aldrich) at a dilution of 1:30,000 for 1 h at 37°C in darkness. Visualization of nitrated groups was carried out using alkaline phosphatase substrate – pNPP, prepared as described for carbonylated protein quantitative measurement. After color development, the reaction was stopped with 5 μl of 5 M KOH and absorbance was read at 405 nm with reference wavelength 605 nm in a microplate reader (Sunrise, Tecan). Measurement of 3-NT proteins were done in three biological replicates, each in three technical replicates and expressed as nmol mg^{-1} protein.

Immunodetection of 3-Nitrotyrosine Proteins

Immunoblotting technique was done for detection of 3-nitrotyrosine proteins. Isolated proteins (as described above) were mixed with sample buffer (described above). After heating at 80°C for 5 min, samples were loaded (soluble protein – amount 25 μg per lane) and SDS-PAGE separated on 10% gels according to Laemmli (1970). An electrotrasfer of proteins to nitrocellulose membrane was done as described for carbonylated proteins. After transfer, proteins were visualized by Ponceau S Red staining. Blocking step was done using 0.1% gelatin in TBST overnight at 4°C. After the washing step nitrocellulose membranes were incubated (1 h, at room temperature in darkness) with monoclonal primary antibodies (Monoclonal Anti-3-Nitrotyrosine antibodies, Sigma-Aldrich; 1:1,000). After washing in TBST (three times), nitrocellulose membranes were covered with secondary antibodies (anti-mouse IgG conjugated with Alkaline Phosphatase Sigma-Aldrich) diluted 1:30,000 for 1 h at room temperature in darkness. Visualization of nitrated groups was carried out as described for carbonylated proteins. Assays were performed in three independent experiments and typical results are shown. The entire gel lanes were quantified by densitometry analysis, which was done using Image J.

POx Activity Measurement

Peroxidase activity measurement was done according to Saunders et al. (1964). Roots of tomato seedling (60 mg) were washed in distilled water, and then homogenized by pestle and mortar in

0.05 M potassium phosphate, pH 7.0 with 10% (v/v) glycerol, 5 mM DTT, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), and 2% (w/v) PVPP in an ice bath. After centrifugation (12,000 *g* for 10 min at 4°C) supernatant was collected for further analyses. Protein extract was incubated with 5 mM pyrogallol in 0.05 M potassium phosphate pH 7.0 at 25°C in darkness. After incubation, POx activity was measured after addition of 1 mM H_2O_2. POx activity was determined as absorbance increase at 430 nm using microplate reader (Sunrise, Tecan), and absorption coefficient $\varepsilon = 2.47\ mM^{-1}\ cm^{-1}$. Results were expressed as nmol $H_2O_2\ min^{-1}\ mg^{-1}$ protein. Experiments were done in at least three biological replicates, each in three technical replicates.

PAO Activity Measurement

Polyamine oxidase activity was measured as described by Luhová et al. (2003) with some modifications. Roots of tomato seedlings (60 mg) after washing in distilled water were homogenized by mortar and pestle in extraction mixture containing 0.05 M potassium-phosphate, pH 6.5, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), 10% (v/v) glycerol, 5 mM DTT, and 2% (w/v) PVPP in an ice bath. The homogenate was centrifuged at 12,000 *g* for 10 min at 4°C. The supernatant was collected for further analyses. PAO activity was measured in 1 ml of reaction mixture containing 0.05 M potassium-phosphate, pH 6.5, 0.5 mM guaiacol, and 1 U of horseradish peroxidase (Sigma-Aldrich) in the presence of 10 mM spermine (Spm). Activity was detected as absorbance increase at 436 nm and calculated using an absorption coefficient $\varepsilon = 25.5\ mM^{-1}\ cm^{-1}$. The results were expressed as nmol $H_2O_2\ min^{-1}\ mg^{-1}$ protein. Experiments were done in four biological replicates, each in three technical replicates.

NADPH Oxidase Activity Measurement

Measurement of NADPH oxidase activity in extracts of tomato roots was done according to Paclet et al. (2007). Tomato roots were isolated, washed in distilled water and homogenized by mortar and pestle in 0.05 M Tris-HCl, pH 7.5 with 0.06 mM Tween-20, 10% (v/v) glycerol, 5 mM DTT, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich), 2% (w/v) PVPP in an ice bath. Homogenates were centrifuged (12,000 *g* for 15 min at 4°C), and supernatants were collected for further analyzes. NADPH oxidase activity was determined by reduction of cytochrome *c*. The reaction mixture contained: supernatant of 50 μg of soluble protein, 0.05 M Tris-HCl, pH 7.5, 60 mM cytochrome *c*, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.1 mM NaCl, 0.06 mM Tween-20. In parallel, cytochrome *c* reduction was done in reaction mixture with addition of 50 U of superoxide dismutase (SOD; Sigma-Aldrich). After incubation at 30°C for 5 min (when $O_2^{\bullet -}$ production from endogenous reductants had ceased) the reaction was started by addition of 0.1 mM reduced β-nicotinamide adenine dinucleotide phosphate (β-NADPH) and cytochrome *c* reduction was measured at 550 nm (Sunrise, Tecan). The presented values were calculated from differences between cytochrome *c* reduction rates in the presence and absence of SOD (Sigma-Aldrich) using an absorption coefficient $\varepsilon = 21.1\ mM^{-1}\ cm^{-1}$. A blank (reaction mixture without supernatant) was also done to every measurement.

The results were expressed as nmol $min^{-1}\ mg^{-1}$ protein. Experiments were done in four biological replicates in three technical replicates.

Measurement of Proteolytic Activity

Determination of proteolytic activity was done according to Pande et al. (2006) with some modifications, using azocasein as a unspecific substrate. After isolation, roots of tomato seedlings (60 mg) were washed in distilled water and homogenized by mortar and pestle in 0.1 M potassium-phosphate, pH 7.0, 0.1% (v/v) Triton X-100, 5 mM DTT, 2% (w/v) PVPP in an ice bath. After two times centrifugation (10,000 *g*, for 5 min, at 4°C), the supernatant was collected, and the protein concentration was measured using Bradford (1976) reagent. Protease activity was measured at pH 5.4 and 8.8. Supernatant containing 20 μg of total soluble proteins was pre-incubated 5 min at 37°C in 0.1 M Tris-HCl, pH 5.4 or 8.8. Subsequently 0.8% (w/v) azocasein solution was added and incubated for 30 min at 37°C in darkness. The reaction was stopped by the addition of 10% (w/v) TCA. Samples were centrifuged (12,000 *g*, for 15 min), supernatant was collected and mixed with 1 M NaOH in 1:1 ratio. After 5 min incubation, the absorbance was measured at 440 nm (Sunrise, Tecan). The specific activity of proteases was expressed as enzyme units per mg protein. Units of enzyme activity were calculated from the absorbance value increase obtained after unspecific protease activity measured in buffers of different pH at 37°C for 30 min as described above. Measurements of proteolytic activity were done in four independent experiments, each in three replicates.

Statistics

Mean values were computed for each experiment and mean differences were calculated using analysis of variance (ANOVA) and Duncan's test. Standard deviation (SE) are also provided to indicate the variations associated with the particular mean values. Calculations were performed using the package agricolae (de Mendiburu, 2010) for the statistical freeware R version 2.14.2 (R Development Core Team, 2012). For growth parameters data were analyzed using the StatGraphics 5.1; Mean ± SE were computed for each experiment and significance of differences was assessed with Student's *t*-test. Differences are considered significant at $p < 0.05$.

RESULTS

CAN Inhibited Elongation Growth of Tomato Roots

Treatment of tomato seedlings with CAN resulted in drastic inhibition of root growth. Inhibition of root growth by 50% was observed in CAN at concentration 10 μM (**Figure 1**), while 50 μM CAN stopped tomato root elongation completely after 72 h (**Figure 1**). Thus, 50 μM CAN was selected as IC_{100} while 10 μM as IC_{50} and used for further investigations.

CAN Enhanced H_2O_2 Concentration and Superoxide Radicals Generation in Roots Extracts of Tomato Plants and Slightly Increased H_2O_2 Efflux from the Roots

Detection of superoxide radicals indicated its stable generation in roots extracts of control seedlings during the culture period (**Table 1**). In extracts of roots of control plants after 24 and 72 h of culture production of superoxide radicals was 1.125 and 1.110 μmol min^{-1} mg^{-1} FW, respectively. In roots extracts of plants treated for 24 h with 50 μM CAN generation of superoxide radicals was similar to the control, while in extracts of plants exposed to 10 μM CAN superoxide radicals generation was twice as high as in the control. Prolonged (for additional 48 h) exposure of seedlings to 10 μM CAN resulted in decline of superoxide radicals generation in roots extracts, but it was still higher than in extracts of seedlings growing in water. High generation of superoxide radicals was detected also in roots extracts of seedlings treated with 50 μM CAN for 72 h, it was twice as high as in the control (**Table 1**).

In roots extracts of control plants H_2O_2 concentration did not differ as culture period was prolonged, and was 75–80 pmol mg^{-1} FW (**Table 1**), equivalent to 78–83 μM. CAN treatment increased H_2O_2 level in roots extracts, leading to a doubling (about 145 μM) after application at higher concentration for 24 h. Longer (72 h) exposure of seedlings to 10 μM CAN enhanced H_2O_2 concentration in roots extracts to around 150% of the control (122 μM). No such effect after 72 h was observed in case of 50 μM CAN. In roots extracts of seedlings treated with 50 μM CAN, H_2O_2 concentration was about 116% of the control (**Table 1**), equivalent to 98 μM.

The concentration of H_2O_2 in the culture medium solution was very low and did not exceed 3.6 nM independently of the treatment. For control plants, during the whole experiment it was stable, and below 1.5 nM (**Table 1**). Expressed per root fresh weigh H_2O_2 content in the medium was significantly higher for plants treated with CAN, and after 24 h reached 461 or 547 pmol g^{-1} FW for 10 or 50 μM CAN respectively, in comparison to control (156 pmol g^{-1} FW). After 72 h culture it declined to 200 or 380 pmol g^{-1} FW for 10 or 50 μM CAN respectively, and 76 pmol g^{-1} FW in control (data not shown).

CAN Inhibited Fluorescence of DAF-FM Derivatives Corresponding to NO Generation and Transiently Enhanced Fluorescence of APF Derivatives Corresponding to $ONOO^-$ Production in Tomato Roots

In roots of control plants growing in water fluorescence of DAF-FM declined slightly (about 25%) during prolonged culture. As expected, CAN inhibited fluorescence of DAF-FM derivatives independently of concentration (**Table 2**). After 24 h fluorescence of DAF-FM derivatives had decreased to 25%, and remained at that level after 72 h.

Fluorescence excitation of APF used for determination of $ONOO^-$ production from control roots did not differ as culture was prolonged. CAN treatment led to slight (30%) and transient enhancement in APF fluorescence after 24 h of exposure (**Table 2**). After 72 h of treatment no differences in fluorescence of APF derivatives for roots of CAN treated seedlings and control were detected.

CAN Treatment Led to Alterations in Concentration of Protein Carbonyl Groups and the Pattern of Carbonylated Soluble Proteins

The level of protein carbonyl groups in homogenates of roots of control plants increased from 30 to 79 nmol mg^{-1} protein, as the culture period was extended for 3 days (**Table 3**). Short term (24 h) exposure to 10 or 50 μM CAN resulted in an increase of the level of carbonyl groups in root homogenates by 53 and 70%, respectively. As CAN treatment was prolonged, the concentration of stable DNP derivatives of the carbonyl groups in proteins isolated from roots increased to 104 nmol mg^{-1} protein in 10 μM CAN or 88 nmol mg^{-1} protein in 50 μM CAN (**Table 3**). In 50 μM CAN it was at the level of the control, while for 10 μM CAN it was 30% higher than in the control.

Detection of total soluble carbonylated proteins isolated from roots of control tomato seedlings separated by SDS-PAGE (**Figure 2A**) and transferred onto a nitrocellulose membrane stained with Ponceau S (**Figure 2B**) for protein transfer

TABLE 1 | Superoxide radicals generation, H_2O_2 concentration in root extracts and H_2O_2 concentration in culture medium of control tomato seedlings and seedlings treated with CAN (10 or 50 μM) for 24 or 72 h.

Plant treatment	Superoxide radicals generation (relative unit)		H_2O_2(nmol g^{-1} FW)		H_2O_2 in culture medium (nM)	
	24 h	72 h	24 h	72 h	24 h	72 h
Control (water)	1.00 ± 0.12c	0.99 ± 0.12c	75.00 ± 5.5c	79.0 ± 6.0c	1.5 ± 0.3c	1.1 ± 0.1c
CAN 10 μM	1.94 ± 0.28a	1.26 ± 0.10b	99.5 ± 10.0b	118.0 ± 12.5a	3.6 ± 0.6a	2.7 ± 0.5ab
CAN 50 μM	1.06 ± 0.22bc	1.89 ± 0.20a	136.5 ± 13.5a	92.0 ± 8.0b	3.4 ± 0.7a	2.3 ± 0.2b

In determination of H_2O_2 concentration in culture medium water or CAN solution of appropriate concentration was used as a blank. One relative unit describing the rate of superoxide radicals generation corresponds to superoxide radicals generation in roots extracts of control plants after 24 h, equal to 1.125 μmol min^{-1} g^{-1} FW. Mean of values are statistically different at $p < 0.05$, when they share no common letter(s). The comparison were made using the Duncan test. Values are average ± SE of 3–4 independent experiments.

TABLE 2 | Fluorescence of DAF-FM derivatives (corresponding to NO) and fluorescence of APF derivatives (corresponding to $ONOO^-$) for roots of tomato seedlings growing in water (control) or roots of seedlings treated with CAN solution at 10 and 50 μM concentration for 24 or 72 h.

Plant treatment	NO (arbitrary units)		$ONOO^-$ (arbitrary units)	
	24 h	72 h	24 h	72 h
Control (water)	0.80 ± 0.07a	0.58 ± 0.06b	0.29 ± 0.06b	0.25 ± 0.06b
CAN 10 μM	0.17 ± 0.02c	0.19 ± 0.02c	0.38 ± 0.06a	0.27 ± 0.05b
CAN 50 μM	0.20 ± 0.02c	0.22 ± 0.03c	0.37 ± 0.07a	0.25 ± 0.05b

Mean of values are statistically different at $p < 0.05$, when they share no common letter(s). The comparison were made using the Duncan test. Values are average ± SE of 3–4 independent experiments.

TABLE 3 | Content of protein carbonyl groups and 3-NT in roots of control tomato seedlings and seedlings treated with CAN (10 and 50 μM) for 24 or 72 h.

Plant treatment	Carbonyl groups (nmol mg^{-1} protein)		3-NT (nmol mg^{-1} protein)	
	24 h	72 h	24 h	72 h
Control (water)	30.0 ± 6.5d	79.0 ± 5.0b	4.2 ± 0.1d	9.4 ± 0.2a
CAN 10 μM	46.0 ± 7.0c	104.0 ± 9.0a	5.5 ± 0.1c	6.8 ± 0.1b
CAN 50 μM	52.0 ± 9.0c	88.0 ± 6.0b	4.9 ± 0.2c	7.0 ± 0.2b

Mean of values are statistically different at $p < 0.05$, when they share no common letter(s). The comparison were made using the Duncan test. Values are average ± SE of at least three independent experiments.

examination showed significant modification in their pattern. In the protein extracts from roots treated with 10 μM CAN for 72 h many carbonylated proteins of molecular weight above 35 kDa were detected. After 72 h of culture thick bands of carbonylated proteins were detected at the top of the membrane corresponding to the top of the gel, both for the control and treated plants (**Figure 2A**). Bands of carbonylated proteins of high density were marked for the control and particularly CAN (50 μM) stressed roots after 72 h, and they were characteristic for proteins corresponding to the band of the molecular marker of 15 kDa. Ponceau S staining of the membrane after protein transfer is shown in **Figure 2B**. In roots of seedlings after 24 h treatment proteins of molecular weight of around 30 kDa were visible, but they were below detection range after 72 h. In contrast more proteins of molecular weight 35–50 kDa were detected in roots of seedlings cultured for 72 h (**Figure 2B**).

CAN Treatment Led to a Slight Decline in Concentration of Total 3-NT and Modification in the Pattern of Nitrated Proteins

For control plants the level of nitrated proteins increased with root age (**Table 3**). It doubled from the value around 4–9 nmol mg^{-1} protein, as the culture period was extended up to 72 h. Short term (24 h) CAN treatment resulted in slight (15–30%) increase in 3-NT concentration in root proteins, as compared to plants growing in water (**Table 3**). As CAN treatment was prolonged, 3-NT concentration increased to 7 nmol mg^{-1} protein, but was 25% lower than that detected in control roots (**Table 3**).

Figures 3A,B shows the protein patterns analyzed using Ponceau S staining and the corresponding tyrosine nitration protein patterns detected with an antibody against 3-NT in roots of control tomato plant or plants exposed to CAN for 24–72 h. In general, roots showed a similar protein tyrosine nitration pattern independently of the treatment, but it differed as experiment was prolonged. Intensity of staining of protein bands below 20 kDa differed in roots of CAN treated plants after 24 h in comparison to control (**Figure 3A**). In roots of seedlings exposed to CAN after 72 h a band of nitrated proteins of 20 kDa is not visible. The nitration profile of proteins isolated from roots after 72 h of the experiment was also different, with less intense band of around 90 kDa being detected. 3-NT-immunopositive proteins of molecular weight above 100 kDa were more abundant in roots after 72 h of the culture both in control and CAN stressed plants (**Figure 3A**).

Activities of Enzymes Involved in Regulation of ROS Level Were Stimulated by CAN

Short-term exposure of plants to CAN did not influence POx activity in roots (**Figure 4A**). In roots of control plants and seedlings treated with CAN (10 and 50 μM) POx activity was of the same range (150–168 nmol min^{-1} mg^{-1} protein). Additional 48 h of culture resulted in enhancement of POx activity both in control and CAN stressed roots. The highest POx activity was observed in roots of seedlings treated with 50 μM CAN, and it was 160% of the control, while 10 μM CAN led to only 15% stimulation of enzyme activity (**Figure 4A**).

During the culture period PAO activity in roots of control seedlings declined from 23 to 16 nmol min^{-1} mg^{-1} protein (**Figure 4B**). CAN treatment resulted in stimulation of PAO activity, after 24 h NPAA influence was concentration independent. In roots growing in CAN for 24 h PAO activity was 60% higher than in control seedlings. Prolongation of CAN stress to 72 h led to further stimulation of enzyme activity. In roots exposed to 10 μM CAN for 72 h PAO activity was doubled as compared to control, while in roots growing in 50 μM CAN its activity was three fold higher than in non-stressed plants (**Figure 4B**).

Activity of NADPH oxidase in roots of plants growing in water was constant during the experiment (**Figure 4C**). CAN treatment stimulated activity of this enzyme. The highest activity of NADPH oxidase, twice as high as the control, was observed after short-term (24 h) application of 10 μM CAN. At 72 h its activity declined, but was still 40% higher than in the control. CAN at concentration of 50 μM led to only slight (15%) stimulation of activity of NADPH

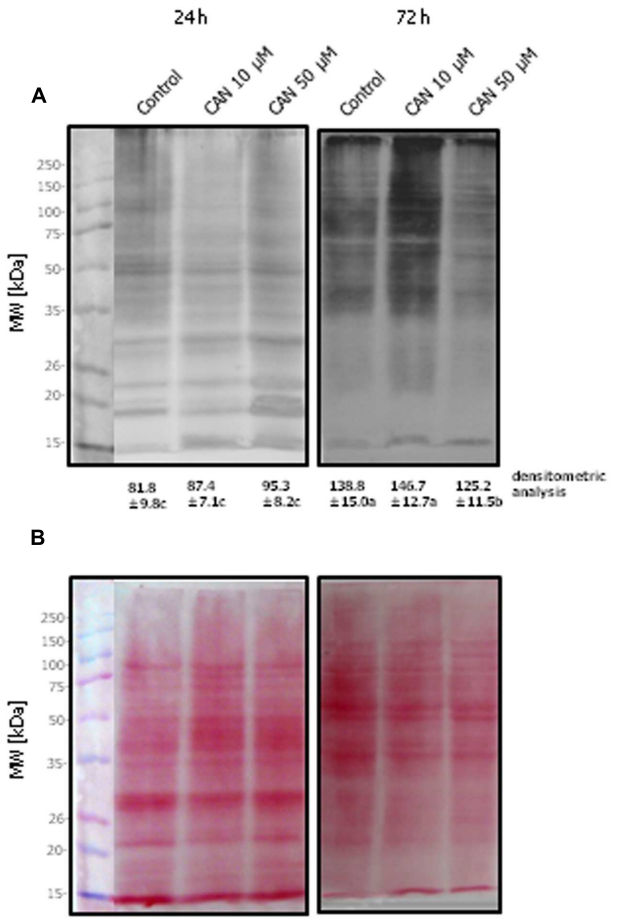

FIGURE 2 | Detection of DNPH-derivatized proteins of tomato roots isolated after 24 or 72 h of culture in water (control) or 10 or 50 μM CAN solution. Total soluble proteins (20 μg per lane) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. **(A)** Oxidized proteins were recognized by anti-DNP antibody. **(B)** The membrane was stained with Ponceau S for examination of protein transfer. The approximate molecular mass for each protein band is indicated in the panels on the left. Experiments were performed three times and representative data are shown. Mean of values of densitometric data are statistically different at $p < 0.05$, when they share no common letter(s). The comparison were made using the Duncan test.

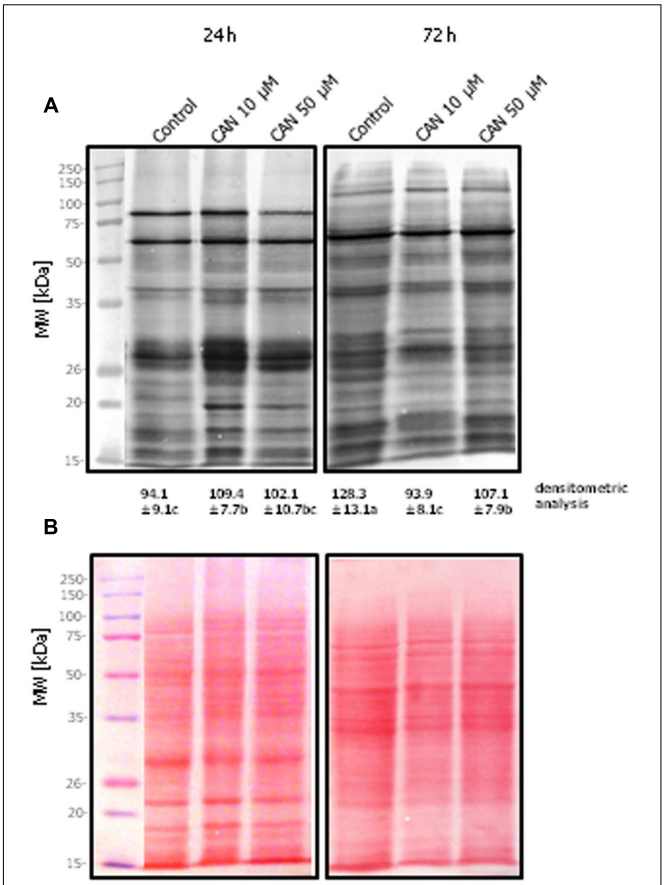

FIGURE 3 | Pattern of 3-NT modified proteins of tomato roots after 24 or 72 h of the culture. Control – plants growing in water, CAN 10 μM-seedlings treated with 10 μM CAN, Can 50 μM – seedlings imbibed in 50 μM Can. Total soluble proteins (25 μg of per lane) were separated by 10% SDS-PAGE and transferred into a nitrocellulose membrane. **(A)** Nitrocellulose after Western blot transfer, followed by detection with monoclonal anti-3-NT antibodies, **(B)** Ponceau S stained nitrocellulose after protein transfer onto membrane. Molecular mass standards in kDa are indicated in the left panels. Experiments were performed 3–4 times and representative data are shown. Mean of values of densitometric data are statistically different at $p < 0.05$, when they share no common letter(s). The comparison were made using the Duncan test.

oxidase at the beginning of the experiment, but after 72 h it was about 80% higher than in plants growing in water (**Figure 4C**).

Proteolytic Activity in Roots of Tomato Seedlings Was Enhanced by CAN Treatment

Proteolytic activity in extract of tomato roots was determined at pH 5.4 and 8.8 (**Figures 5A,B**). In roots of control plants after 24 h of growth, proteolytic activity was around 0.3 U mg^{-1} protein at both pH. After 72 h it doubled at acidic pH, and was stable at basic pH. Treatment of seedlings with CAN led to stimulation of protolytic activity at both pH. The highest proteolytic activity was observed at pH 5.4 after 72 h of culture (**Figure 5A**).

DISCUSSION

Canavanine is a natural NPAA produced by some plants. As a compound present in seeds or leaves it could be consumed by animals or people. Due to antiviral, antifungal, antibacterial, and anticancer properties CAN is used in medicine (Ekanayake et al., 2007). Toxicity of CAN was shown for herbivores (Rosenthal, 2001; Jang et al., 2002), but also for plants, including apple (*Malus domestica* Borkh.) embryos (Krasuska et al., 2014a). Just recently, we demonstrated that the presence of CAN in the growing medium of young (3–4 days-old) tomato seedlings

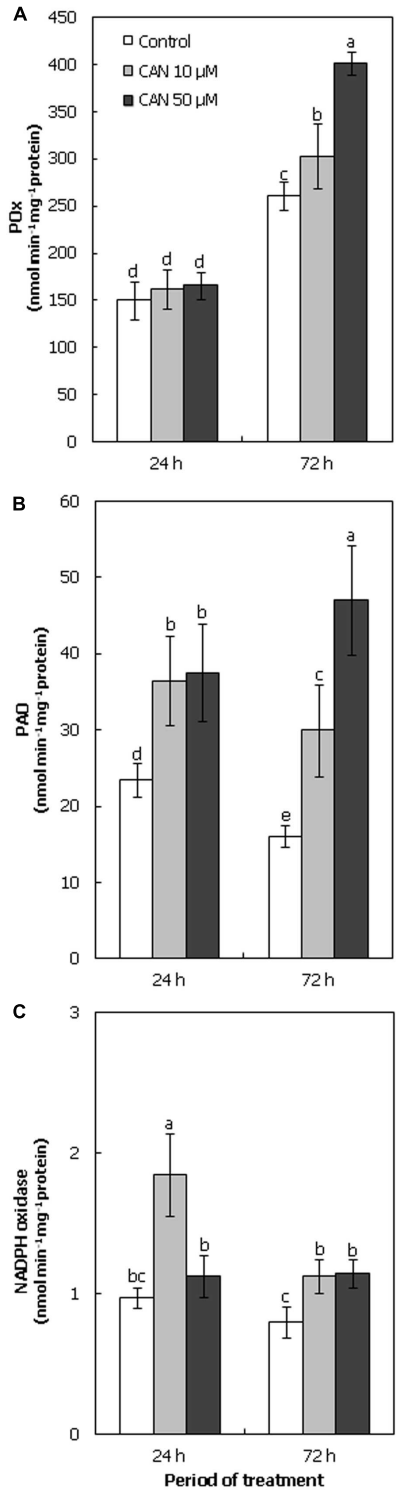

FIGURE 4 | Activity of POx (A), PAO (B), and NADPH oxidase (C; nmol min^{-1} mg^{-1} protein) in roots of tomato seedlings growing in water (control) or treated with CAN (10 or 50 μM) after 24 or 72 h of culture period. Mean of values are statistically different at $p < 0.05$, when they share no common letter(s). The comparison were made using the Duncan test. Values are average ± SE of at least three independent experiments and three biological repetitions each.

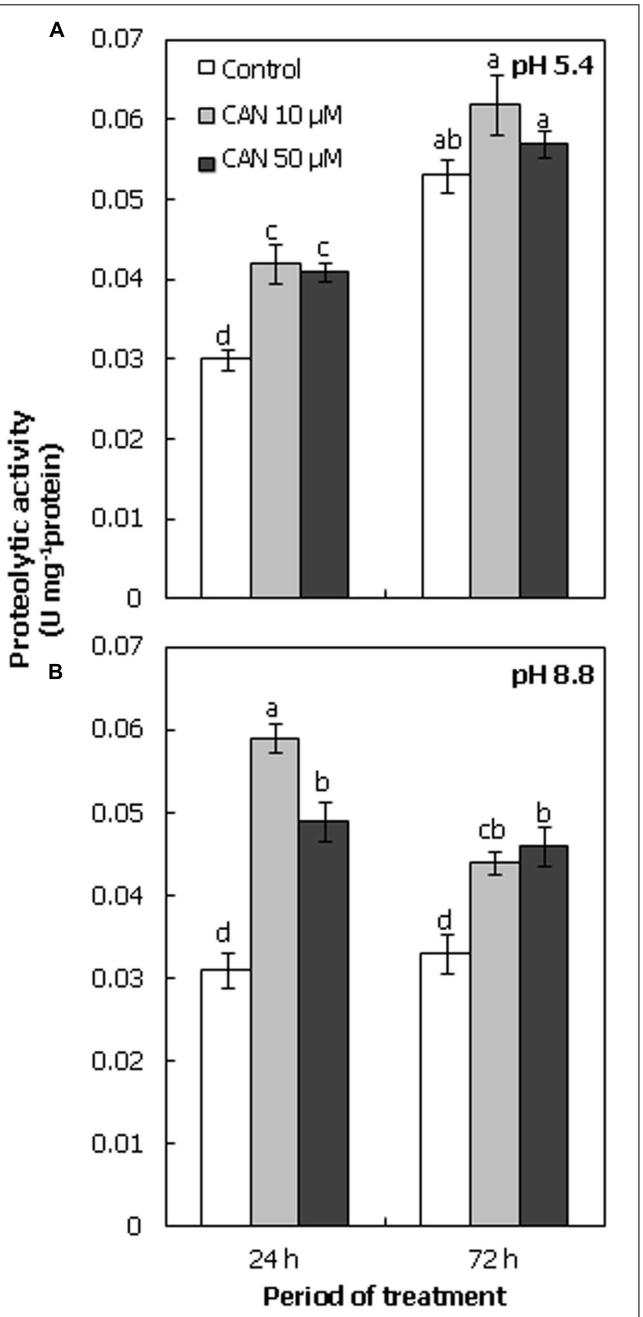

FIGURE 5 | Proteolytic activity (U mg^{-1} protein) in roots of tomato seedlings cultured for 24 or 72 h in water (control) or in 10, 50 μM CAN solution, determined at acidic pH (5.4; A) or at basic pH (8.8; B). Mean of values are statistically different at $p < 0.05$, when they share no common letter(s). The comparison were made using the Duncan test. Mean ± SE, $n = 10$–12.

led to an inhibition of root elongation growth. This effect was dose- and time-dependent (Krasuska et al., 2016). CAN is a substrate of arginyl-tRNA synthetases responsible for the incorporation of NPAA instead of Arg into a protein structure (Jang et al., 2002). Occurrence of dysfunctional proteins leads to a disruption of metabolism and finally stress induction. Common

physiological reaction to abiotic or biotic stressors is ROS/RNS burst (imbalance in ROS/RNS production and scavenging; Groß et al., 2013). It has also been noticed as secondary mode of action of some allelochemicals or phytotoxins (Gniazdowska et al., 2015).

We have shown that CAN, starting from 24 h of treatment, stimulated superoxide radicals generation (**Table 1**), mostly generation of superoxide anion ($O_2^{\bullet -}$), as we reported previously using root staining with NBT and correlated well to O_2 consumption (Krasuska et al., 2016). CAN enhanced also H_2O_2 concentration in root extracts of tomato seedling (**Table 1**). It was accompanied by the exudation of H_2O_2, however, CAN did not disturb membrane permeability and roots viability (Krasuska et al., 2016). Thus, ROS exudation into the environment could be a root response to NPAA. The rise of ROS level and radicals production in root extracts of tomato seedlings exposed to CAN was comparable to those detected in *Arabidopsis* plants grown in the medium supplied with *p*-hydroxybenzoic acid (Guan et al., 2014) and to those in onion (*Allium cepa* L.) or maize (*Zea mays* L.) treated with cyanamide, an allelochemical of hairy vetch (*Vicia villosa* Roth.; Soltys et al., 2011, 2014). Enhanced $O_2^{\bullet -}$ level was observed in cyanobacteria *Microcystis aeruginosa* (Kützing) after berberine application, and associated with growth inhibition (Zhang et al., 2011). Disruption of $O_2^{\bullet -}$ and H_2O_2 ratio in root meristem resulted in restriction of root cells growth (Tsukagoshi et al., 2010). Therefore, inhibition of elongation of the tomato root by CAN could be explained by ROS-dependent disorder of cell differentiation or proliferation, which could be the secondary mode of action of this NPAA. The incubation of mouse glial cell lines with CAN resulted in an enhancement of ROS content, accompanied by a decline in the reduced form of glutathione (Riganti et al., 2003). CAN also stimulated the pentose phosphate pathway, a strong marker of oxidative stress. Moreover, in a cell-free system, CAN decreased DTT level (Riganti et al., 2003), indicating that this NPAA could take part in the regulation of redox state of small thiol containing compounds.

Canavanine lowered the fluorescence rate of DAF-FM, corresponding to NO formation in the tomato seedling roots (**Table 2**), similarly, as was detected previously for root tips (Krasuska et al., 2016). This effect seems to be uncommon, as stress factors usually induce secondary nitro-oxidative stress. On the other hand, it suggests that CAN could inhibit or disturb formation of NO *via* still unidentified Arg-dependent pathway, which seems to be important for the maintenance of regular root growth. In our experiment, a slight increase in fluorescence of APF, corresponding to $ONOO^-$ production in tomato roots was observed only after 24 h of the culture in CAN solution (**Table 2**) and matches well to microscopic localization of $ONOO^-$ in root tip cells (Krasuska et al., 2016). As compared to short-term CAN treatment, prolonged (72 h) exposure to CAN resulted in a decline in the $ONOO^-$ generation. The transient enhancement of $ONOO^-$, measured using APF, can be linked to an elevated production of ROS (particularly $OH^{\bullet}$).

The increase in the content of protein carbonyl groups is a commonly accepted marker for ROS imbalance. There is no information about the impact of allelopathic compounds on the level of protein carbonyl groups in tissues of acceptor plants. The treatment of tomato seedlings with CAN led to an increased concentration of carbonyl groups (**Table 3**). In CAN treated roots higher abundance of carbonylated proteins was especially observed after 72 h (**Figure 2**). Observing a band on the membrane corresponding to the front of the gel we can conclude that CAN led to increased abundance of carbonylated proteins of low molecular weight. According to the concept of Møller and Sweetlove (2010) some carbonylated peptides released from proteins, after their breakdown, can serve as signaling molecules of specific cellular compartment origin. Previously, it was demonstrated that several storage proteins, in seeds are carbonylated to accelerate their mobilization during the germination process (Job et al., 2005; Krasuska et al., 2014b). In high abundance, we have also detected carbonylated proteins that were not entering the gel. These were probably aggregates of various proteins, content of which increased after 72 h of the culture, both in CAN treated and control plants (**Figure 2**). Protein aggregates may diminish their ability for degradation (Jung et al., 2007). The physiological explanation for the accumulation of aggregates is hard to clarify, however, such phenomenon occurs also in regularly growing roots. Differences between quantitative detection of carbonylated proteins (**Table 3**) and results of Western blot assay (**Figure 2**) were due to absorption of proteins and/or fragments of proteins on Maxisorp plates. Maxisorp plates bind also small proteins, which can be lost in protein transfer during Western blot analysis. It could be also suspected that due to high proteolytic activity after treatment with CAN (**Figure 5**) fragments of small proteins, which were bonded to the surface of the plates were lost in Western blot.

During the progression of the leaves vegetative development in *Arabidopsis* cultured under optimal conditions, an increase of the level of carbonylated proteins was detected (Johansson et al., 2004). Similarly, we observed that the alterations in the level of protein DNP derivatives in roots of the control tomato seedling, during the progression of growth, were of the same pattern as described in the roots of apple seedlings (Krasuska et al., 2014b). The content of carbonyl groups in tomato roots at the beginning of the experiment (roots were of length of 5 mm) was higher (data not shown) than in the roots of plants growing in water for additional 24 h. During the extension of root elongation growth the amount of carbonyl groups in proteins doubled (**Table 3**). Alike, in roots of apple embryos at the termination of germination *sensu stricto* higher level of carbonylated peptides, than in the roots of 10-days-old seedlings cultured in water, was noticed (Krasuska et al., 2014b). Thus, it implies that accumulation of some carbonylated proteins could be due to physiological processes occurring during growth and development, not simply related to the risks of oxidative stress.

Bearing in mind that ROS act as key molecules in the regulation of root growth and development, specific carbonylated proteins may be involved is signal transduction. There is also a possibility, that carbonylation could serve as a protective mechanism, that addresses/directs putative aberrant proteins enriched in CAN to faster degradation. Isolation and identification of such proteins would be of great interest to explain CAN toxicity. The more, in HEp-2 cells it was shown that endogenous canavanyl proteins are preferentially ubiquitinated

and probably processed to degradation via proteasome (Akaogi et al., 2006).

The mode of action of RNS involves a modification of protein amino acid residues such as, e.g., Tyr (Astier and Lindermayr, 2012). Following disturbances in RNS level after CAN application we measured the 3-NT level in proteins isolated from roots of tomato seedlings (**Table 3**). Just like the content of protein carbonyl groups points at ROS metabolism, amplification of Tyr nitration can serve as a marker of nitrosative stress. CAN treatment led to a slight increase followed by a decline of 3-NT in proteins in roots of tomato seedlings (**Table 3**). The increase in the 3-NT level after short term CAN treatment is in agreement with the data obtained for $ONOO^-$ production, and can also be due to the higher abundance of small proteins. The lower (in comparison to the control) level of 3-NT groups after prolonged CAN supplementation indicated that NO cellular production was limited, or NO production was unaffected, but the reaction with $O_2^{\bullet -}$ was accelerated. Increase of $O_2^{\bullet -}$ (free radical burst) can be also a reason of lowering cellular NO concentration, as these compounds react rapidly. On the other hand, we have demonstrated equal concentration of NO_2^- in CAN stressed and non-treated tomato seedling (Krasuska et al., 2016). $ONOO^-$ is not the only one nitration agent, as this process occurs also in the presence of NO_2^- (Speckmann et al., 2016). Thus, we suspect that CAN influences other mechanisms of NO generation (probably enzymatic pathways), being beneficial during root growth and development.

Summing up these results with those of NO efflux we can conclude that CAN serves rather as inhibitor of RNS burst, and in consequence, may have a stressful effect. Therefore, the restriction of root growth in tomato seedlings by CAN could be linked to the disturbances in RNS tissue level. NO is required for an undisturbed root growth (Yu et al., 2014). Corpas et al. (2009) indicated that in roots of 71-days-old pea (*Pisum sativum* L.) plants growing under optimal conditions proteins with 3-NT were present at a level higher than in other organs, e.g., leaves or flowers. So, protein nitration is of physiological occurrence, similarly as we previously pointed to carbonylation. In 2-weeks-old seedlings of *Arabidopsis* more than 60% of the identified nitrated proteins were engaged in primary metabolism (Lozano-Juste et al., 2011), suggesting that inhibition of this PTM and/or deficiency of specific nitrated proteins could disrupt cellular homeostasis. We can also assume that peptides/proteins with 3-NT could serve as signaling molecules of specify nature and origin. Our data indicates that CAN treatment destabilizes this potent 3-NT-dependent signaling pathway. Tyr nitration increased during various stress conditions (Corpas et al., 2008). Stress factors led to a substantial strengthening of protein bands immunolabeled against 3-NT of molecular weight of 29–59 kDa isolated from pea plants. The treatment of tomato roots with CAN led to a transient (in first 24 h) increase of the intensity of 3-NT-labeling of 20 and 30 kDa protein band, while in the control samples these bands were almost at the same level up to 72 h (**Figure 3**). These proteins are probably associated with the phytotoxicity of CAN, and thus needs identification.

Canavanine treatment of tomato seedlings increased proteolytic activity (**Figure 5**). Moreover, the transient (observed after 24 h) increase in protein nitration and carbonylation (compared to the control) could favor their proteolysis. Protein nitration enhances the susceptibility to degradation by proteasome pathway, on the other hand oxidative/nitrosative stress conditions lead to an inactivation of proteasome (Souza et al., 2000; Kästle et al., 2012). Furthermore, it was shown that at high cellular ROS level oxidatively modified proteins were degraded by peroxisomal proteases (Romero-Puertas et al., 2002). In addition, proteases participate in the cellular defense system (Fowler et al., 2009), so we can conclude that a higher proteolytic activity after plant treatment with CAN is part of the detoxification machinery. Aberrant products of CAN incorporation into mitochondrial proteins of human cells lines were detected only for a short time, suggesting their enhanced degradation (Konovalova et al., 2015). Similarly, the decrease in the content of carbonylated proteins during apple embryo germination, the process stimulated by NO or hydrogen cyanide (HCN), was linked to a stimulation of proteolytic activity (Dębska et al., 2013; Krasuska et al., 2014b).

As mechanical properties of cell wall depend on ROS interaction with cell wall components, inhibition of root elongation of tomato seedlings observed after CAN treatment could be connected to the destabilization of cell wall loosening – stiffening process. We focused on the activity of ROS producing enzymes: PAO, POx, and NADPH oxidases (**Figure 4**). PAOs catalyze oxidative degradation of PAs: spermine (Spm) and spermidine (Spd), polycationic compounds belonging to the group of plant growth and development regulators. Co-product of this reaction is H_2O_2 (Kusano et al., 2008). Supplementation with CAN stimulated PAO activity in tomato roots, especially after 24 h of the culture (**Figure 4**), suggesting involvement of CAN in PAs metabolism. PAs take part in plant responses to various stress factors, including allelochemicals (Kusano et al., 2008; Gniazdowska et al., 2015). 2(3H)-benzoxazolinone decreased PAs content in roots of lettuce (*Lactuca sativa* L.; Sánchez-Moreiras and Reigosa, 2005). Cinamic acid reduced Spm and Spd levels in leaves of cowpea [*Vigna unguiculata* (L.) Walp.], which could be explained by the stimulation of PAO activity. Moreover, it was accompanied by an induction of oxidative stress (Huang and Bie, 2010).

Other enzymes involved in ROS metabolism are POx, especially extracellular heme-containing Class III peroxidases. The activity of POx of apoplastic localization is connected with cell wall stiffening by participation in lignin production (Liszkay et al., 2003, 2004). As was mentioned above, CAN treatment of tomato plants resulted in an inhibition of elongation growth of roots, which in addition became visibly thicker than the control. The higher POx activity in roots of seedlings treated with CAN, observed especially after 72 h of the culture (**Figure 4**), can be linked to lignin production and root stiffening. Up-regulation of the gene coding POx accompanied by an elevated level of ROS was noted in plants treated with gallic acid (Rudrappa et al., 2007; Golisz et al., 2008), which indicates that this enzyme has a function in plant response to phytotoxins. Oxygen free radicals are products of NADPH oxidases, the activity of which is coupled with the regulation of growth of root tip cells (Foreman et al., 2003), and response to allelochemicals

(Oracz et al., 2012). Cinnamic acid increased ROS production, accompanied by the stimulation of NADPH oxidase activity in roots of cucumber seedlings (Ding et al., 2007). In our experiment CAN supplementation enhanced the activity of NADPH oxidases in tomato roots (**Figure 4**). These results correspond well with transiently elevated $ONOO^-$ and 3-NT level in proteins in roots treated with CAN. An increase in NADPH oxidase activity pointed, that indeed $O_2^{\bullet -}$ production after CAN application was greater and higher $O_2^{\bullet -}$ level was not due to lower NO and its lower conversion to $ONOO^-$. In contrast, the reduced expression of genes encoding NADPH oxidase in transgenic cress (*Lepidium sativum* L.) seedling resulted in inhibition of root length (Müller et al., 2012).

Canavanine disrupts vegetative growth and development of young tomato seedlings and inhibits elongation of the primary root. CAN acts as stress factor, leading to an overproduction of ROS. In addition, CAN dependent stress generation in plant tissue is also due to a decline in the cell's RNS formation, resulting in the maintenance of RNS concentration at the level below its physiological rate. Our data indicates that the incorporation of CAN into proteins is not the only origin/base of CAN phytotoxicity. By imbalance in ROS/RNS CAN impacts protein PTMs, thus almost certainly affects the primary metabolism. Taking into account the results of the presented experiments, it would be of great importance to identify the aberrant CAN incorporated proteins, and verify their sensitivity toward redox dependent PTMs.

AUTHOR CONTRIBUTIONS

The experiments were conceived and designed by: UK and AG, RB helped in research discussion. The experiments were performed by: OA, PS, and UK. The data were analyzed by: UK, AG, OA, and PS. The paper was written by: UK and AG. All authors read and approved the final version of the manuscript.

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MINI REVIEW
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Antioxidant Systems are Regulated by Nitric Oxide-Mediated Post-translational Modifications (NO-PTMs)

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Nitric oxide (NO) is a biological messenger that orchestrates a plethora of plant functions, mainly through post-translational modifications (PTMs) such as *S*-nitrosylation or tyrosine nitration. In plants, hundreds of proteins have been identified as potential targets of these NO-PTMs under physiological and stress conditions indicating the relevance of NO in plant-signaling mechanisms. Among these NO protein targets, there are different antioxidant enzymes involved in the control of reactive oxygen species (ROS), such as H_2O_2, which is also a signal molecule. This highlights the close relationship between ROS/NO signaling pathways. The major plant antioxidant enzymes, including catalase, superoxide dismutases (SODs) peroxiredoxins (Prx) and all the enzymatic components of the ascorbate-glutathione (Asa-GSH) cycle, have been shown to be modulated to different degrees by NO-PTMs. This mini-review will update the recent knowledge concerning the interaction of NO with these antioxidant enzymes, with a special focus on the components of the Asa-GSH cycle and their physiological relevance.

Keywords: ascorbate-glutathione cycle, catalase, superoxide dismutase, peroxiredoxin, nitric oxide, S-nitrosylation, tyrosine nitration

INTRODUCTION

Nitric oxide is a gaseous molecule and a pivotal biological messenger. NO is involved in signaling pathways that are related to fundamental processes in plant biology such as growth and development (Beligni and Lamattina, 2000; Pagnussat et al., 2002), senescence (Begara-Morales et al., 2013) and response to abiotic (Corpas et al., 2011; Siddiqui et al., 2011) or biotic stress (Delledonne et al., 1998; Durner et al., 1998; Feechan et al., 2005). Generally, the rise in NO levels in response to stress conditions is accompanied by another group of molecules called reactive oxygen species (ROS), some of which, particularly H_2O_2, are also involved in multiple signaling pathways

Abbreviations: Asa-GSH cycle, ascorbate-glutathione cycle; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; MDAR, monodehydroascorbate reductase; NO-PTMs, nitric oxide-related post-translational modifications.

(Neill et al., 2002). This mini-review will explore recent findings concerning the modulation of the main antioxidant enzymes by NO, especially the enzymatic components of Asa-GSH cycle, with particular attention to the molecular mechanism underpinning this key regulatory pathway in response to stress situations.

NITRIC OXIDE-MEDIATED POST-TRANSLATIONAL MODIFICATIONS: NITRATION AND *S*-NITROSYLATION

Nitric oxide mainly transmits its action via post-translational modifications, such as *S*-nitrosylation and tyrosine nitration, which can regulate the function of the target proteins (Astier and Lindermayr, 2012). These NO-PTMs may be involved in cell signaling under physiological and stress conditions (Corpas et al., 2015).

Tyrosine nitration, which is mediated mainly by peroxynitrite ($ONOO^-$), consists of the addition of NO_2 radicals to one of the two equivalent ortho-carbons of the aromatic ring of tyrosine residues leading to 3-nitrotyrosine (Gow et al., 2004; Radi, 2004). This modification converts the tyrosine into a negatively charged residue and causes a marked shift in the hydroxyl group's pKa (Turko and Murad, 2002; Abello et al., 2009) which can affect the target proteins resulting in a gain, loss or no change in the protein's function (Souza et al., 2008; Radi, 2013). Although tyrosine nitration has been traditionally considered as an irreversible mechanism and a nitrosative stress marker, the existence of tyrosine denitrase activity that reduces 3-nitrotyrosine in mammalian cells (Görg et al., 2007; Deeb et al., 2013) pointing toward a role of tyrosine nitration in NO-mediated signaling processes in these cells.

S-nitrosylation consists of the addition of a NO group to a cysteine thiol leading to *S*-nitrosothiols (SNOs) and consequently can also alter the function of a broad variety of proteins (Hess et al., 2005; Astier et al., 2011). *S*-nitrosoglutathione (GSNO), formed by *S*-nitrosylation of the antioxidant GSH, is the major low-molecular-weight *S*-nitrosothiol. It is considered to be a NO reservoir in cells (Gaston et al., 1993; Durner et al., 1999; Leitner et al., 2009) that due to its phloem mobility is involved in signaling mechanisms. Furthermore, GSNO can mediate transnitrosylation reactions in which a new *S*-nitrosothiol is generated by transferring its NO group to a new cysteine thiol group (Hess et al., 2005).

S-nitrosylation is a reversible mechanism since SNO can be specifically and enzymatically broken down by thioredoxins (Benhar et al., 2008; Kneeshaw et al., 2014), in addition to the non-enzymatic decomposition by antioxidants such as ascorbate or glutathione. Furthermore, *S*-nitrosoglutathione reductase (GSNOR) decomposes GSNO and indirectly controls SNO levels (Liu et al., 2001; Feechan et al., 2005).

In recent years, mounting evidence has shown that SNOs are fundamental players in NO-signaling pathways in plant biology (Belenghi et al., 2007; Romero-Puertas et al., 2007, 2008; Lindermayr and Durner, 2009; Astier et al., 2011; Hu et al., 2015), with an important role in plant immunity and plant response to abiotic stresses (Feechan et al., 2005; Rusterucci et al., 2007; Valderrama et al., 2007; Corpas et al., 2008; Chaki et al., 2009a, 2011a,b). Due to its importance, increased efforts have been made to identify the processes that could be regulated by SNOs and subsequently hundreds of proteins that undergo *S*-nitrosylation under physiological or adverse conditions have been identified over the past decade.

S-NITROSYLATION CONTROLS $ONOO^-$ LEVELS VIA REGULATION OF PrxII E

Peroxiredoxins (Prx) are thiol based peroxidases that can be involved in multiple functions in addition to its role in detoxifying H_2O_2 (for review see Bhatt and Tripathi, 2011). Some Prxs have been identified to be regulated by NO-PTMs in animals and plants. In mammals, *S*-nitrosylation inhibits the enzymatic activity of neuronal Prx2 (Fang et al., 2007) and Prx1 (Engelman et al., 2013) whereas the peroxidase activity of Prx2 from mammalian erythrocytes was induced after tyrosine nitration (Randall et al., 2014). In plants, *S*-nitrosylation inhibits the peroxidase activity of PrxII E (Romero-Puertas et al., 2007) and PrxII F (Camejo et al., 2015). Interestingly, some members of Prx family posses $ONOO^-$ reductase activity (Bryk et al., 2000; Romero-Puertas et al., 2007; Pedrajas et al., 2010) and therefore could protect against $ONOO^-$-mediated oxidative and nitrosative stresses. In plants, PrxII E is *S*-nitrosylated during hypersensitive response (Romero-Puertas et al., 2008) and this modification inhibits its peroxynitrite reductase activity promoting tyrosine nitration (Romero-Puertas et al., 2007). Therefore, *S*-nitrosylation emerges as a key mechanism in $ONOO^-$ homeostasis, regulating endogenous level of $ONOO^-$ and tyrosine nitration via control of PrxII E (Romero-Puertas et al., 2007). Changes in $ONOO^-$ levels and/or tyrosine nitration have been related to several abiotic/biotic stresses (Valderrama et al., 2007; Corpas et al., 2008; Chaki et al., 2009a, 2011a,b). Consequently, understanding if *S*-nitrosylation of PrxII E could be involved in plant response to these stress conditions is a good issue to be addressed in the future.

NITRIC OXIDE INTERACTIONS WITH CATALASE AND SUPEROXIDE DISMUTASES

Superoxide dismutase (SOD) is a group of metalloenzymes that catalyze the disproportionation of superoxide radicals into H_2O_2 (Fridovich, 1986; Halliwell and Gutteridge, 2000). SODs are classified into three main types containing Mn, Fe, or Cu plus Zn as prosthetic metals and they are present in all cell compartments (Parker et al., 1984; Zelko et al., 2002). In eukaryotic cells from different organisms, it has been demonstrated that Mn-, Fe-, and CuZn-SODs undergo inactivation by peroxynitrite-mediated nitration (Demicheli et al., 2007; Martinez et al., 2014) and SOD activity is increased after GSNO treatment (Sehrawat et al., 2013). Recently, *in vitro* approaches have been used to analyze the

effect of NO-mediated PTMs on the different SOD isozymes in *Arabidopsis thaliana*. Thus, whereas *S*-nitrosylation did not affect SOD activities, nitration inhibited Mn-SOD1, Fe-SOD3, and CuZn-SOD3 activity to different degrees but affected no other SOD isozymes (Holzmeister et al., 2015).

On the other hand, catalase, which is a peroxisomal key enzyme that regulates H_2O_2 levels (Chance et al., 1979; Kirkman and Gaetani, 1984), was one of the first antioxidant enzymes to be analyzed *in vitro* to check how its activity can be modulated by NO donors (Clark et al., 2000). At present, it is known that plant catalase can be nitrated and *S*-nitrosylated *in vitro,* both of which inhibit its activity (Clark et al., 2000; Ortega-Galisteo et al., 2012), although, according to the literature available, the specific target residues have not yet been identified. Very recently, it has been determined by proteomic approaches that catalase undergoes increasing nitration during pepper fruit maturation, decreasing its activity as consequence of potential tyrosine nitration as corroborated after treatment with SIN-1 (a peroxynitrite donor; Chaki et al., 2015). This inhibition could imply a lower capacity for removing H_2O_2 and therefore is well correlated with the increase of the oxidative metabolism observed during this physiological process (Martí et al., 2011; Chaki et al., 2015).

ASCORBATE-GLUTATHIONE CYCLE AND NITRIC OXIDE-PTMs

Ascorbate-glutathione cycle is a pivotal antioxidant system involved in the regulation of H_2O_2 levels (Asada, 1992; Noctor and Foyer, 1998; Shigeoka et al., 2002) under development and unfavorable conditions in plant cells. The cycle is composed of the enzymes APX, MDAR, DHAR, and GR plus the non-enzymatic antioxidants ascorbate and glutathione (GSH). Concomitant to H_2O_2 reduction to water, APX catalyzes the oxidation of ascorbate to monodehydroascorbate (MDA) which can spontaneously generate dehydroascorbate (DHA). Ascorbate is regenerated by MDAR and DHAR using NADPH and GSH as electron donors, respectively. Finally, GR is involved in regenerating GSH levels.

Analyzing how NO regulates Asa-GSH cycle is a key issue to understand the interplay between NO and antioxidant systems (**Figure 1**). In this sense, enzymatic activity of the components of Asa-GSH cycle can be modulated by NO and under different stress situations (Groβ et al., 2013). Additionally, these enzymes have been identified as targets of NO-PTMs, identifying in some cases the molecular mechanism involved in these modifications (**Table 1**).

Regulation of Asa-GSH Cycle by Tyrosine Nitration

Proteomic approaches have identified all enzymes of the Asa-GSH cycle as potential nitrated proteins (Chaki et al., 2009b; Lin et al., 2012; Tanou et al., 2012). However, information related to the specific impact of this modification on the structure of these target proteins and the role of the tyrosine target of nitration is necessary in order to understand the cross-talk between NO and ROS in the antioxidant defense against nitrosative stress. In this respect, two recent studies have identified the tyrosine target(s) of nitration and its (their) potential role within the mechanistic activity of the Asa-GSH cycle enzymes, showing that this NO-PTM could compromise the Asa-GSH cycle functioning (Begara-Morales et al., 2014, 2015). Pea cytosolic APX is inactivated by $ONOO^-$ as consequence of tyrosine nitration (Begara-Morales et al., 2014) and as result the detoxification of H_2O_2 by Asa-GSH cycle could be compromised (**Figure 1**). Proteomics and *in*

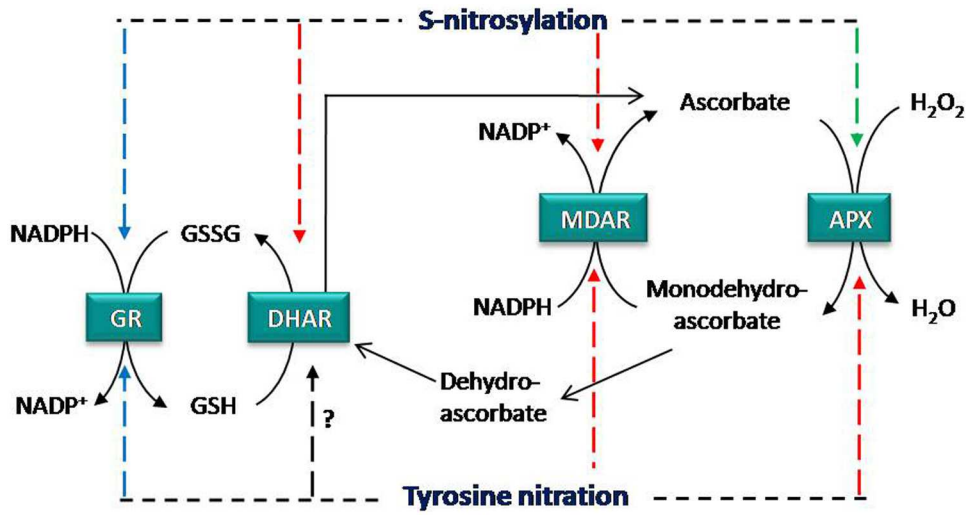

FIGURE 1 | Regulation of ascorbate glutathione cycle by NO-PTMs. APX activity is inhibited by tyrosine nitration and enhanced by *S*-nitrosylation, whereas MDAR is inhibited by both NO-PTMs. DHAR is inhibited by *S*-nitrosylation and GR is not affected by these PTMs. Color of arrows shows the effect of tyrosine nitration and *S*-nitrosylation on enzymatic activities; red: inhibition, green: enhancement, and blue: no effect. APX, ascorbate peroxidase; MDAR, monodehydroascrobate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase.

TABLE 1 | Effect of NO-PTMs on components of Asa-GSH cycle.

Protein	NO-PTM	Effects	Target	Plant species	Reference
Ascorbate peroxidase (APX)	Tyrosine nitration *S*-nitrosylation	Decreased activity Increased activity	Tyr235[(1)] Cys32[(1)(2)(3)]	*Pisum sativum* *Arabidopsis thaliana, Pisum sativum*	Begara-Morales et al., 2014 Begara-Morales et al., 2014; Yang et al., 2015
Monodehydro-ascorbate reductase (MDAR)	Tyrosine nitration *S*-nitrosylation	Decreased activity Decreased activity	Tyr345[(1)] Cys68[(3)]	*Pisum sativum* *Pisum sativum*	Begara-Morales et al., 2015 Begara-Morales et al., 2015
Dehydro-ascorbate reductase (DHAR)	Tyrosine nitration *S*-nitrosylation	N.D. Decreased activity	N.D. Cys20[(1)(2)], Cys147[(1)(2)]	N.D. *Arabidopsis thaliana; Solanum tuberosum*	Fares et al., 2011; Kato et al., 2013; Puyaubert et al., 2014
Glutathione reductase (GR)	Tyrosine nitration *S*-nitrosylation	No effect	N.D.	*Pisum sativum*	Begara-Morales et al., 2015

Nitration and S-nitrosylation targets have been identified by different technological approaches: (1) Mass spectrometry, (2) site-directed mutagenesis, and (3) in silico identification. ND: Not determined.

silico approaches identified the Tyr235 as the most reliable target responsible for APX inactivation, since this residue is located just at 3.6 Å from the heme group at the bottom of the catalytic pocket (Patterson and Poulos, 1995; Jespersen et al., 1997; Mandelman et al., 1998; Begara-Morales et al., 2014). Consequently, Tyr235 nitration may disrupt heme-group properties and result in a loss of activity (Begara-Morales et al., 2014).

Monodehydroascorbate reductase, which is involved in the regeneration of ascorbate (**Figure 1**), is another significant modulation point of the Asa-GSH cycle. In this case, $ONOO^-$ mediates nitration of recombinant pea MDAR at Tyr213, Tyr292, and Tyr345, causing an inhibition of the enzymatic activity (Begara-Morales et al., 2015), and therefore may disrupt the regeneration of ascorbate and compromise the functioning of the Asa-GSH cycle. Site-directed mutagenesis demonstrates that Tyr345 is the main residue responsible for the loss of activity after nitration, since this tyrosine is located just at 3.3 Å from His313, which is involved in NADP binding, suggesting that the nitration of this tyrosine could alter the positioning of the cofactor, thereby decreasing protein activity (Begara-Morales et al., 2015). DHAR is the other enzyme involved in the regeneration of ascorbate, but, although DHAR has been reported to be nitrated (Tanou et al., 2012) and its activity modulated by NO (see Groβ et al., 2013), no information is available on the tyrosine(s) involved in this modification and the impact on the protein structure.

Glutathione reductase has also been identified as tyrosine nitration target (Chaki et al., 2009b). In animals, peroxynitrite inhibits human and bovine GR activity by nitration of Tyr106 and Tyr114 which are located close to the GSSG binding zone (Francescutti et al., 1996; Savvides et al., 2002). However, very recently and in contrast to animals, it has been strikingly shown that chloroplastic and cytosolic pea GR activities are not affected by peroxynitrite-mediated tyrosine nitration (Begara-Morales et al., 2015). This behavior is unusual in higher plants, where the main effect of tyrosine nitration on target proteins is usually a loss of function (Astier and Lindermayr, 2012; Begara-Morales et al., 2013; Chaki et al., 2013; Corpas et al., 2013).

S-Nitrosylation on Asa-GSH Cycle

S-nitrosylation has emerged as a key PTM that regulates fundamental processes in plant biology such as plant immunity or plant response to (a)biotic stress. Consequently, this PTM has become the main pathway by which NO acts as a signaling molecule. Hundreds of proteins related to a wide range of metabolic pathways have been reported to be *S*-nitrosylated in plants. For instance, NO regulates many enzymes involved in ROS/RNS generation/scavenging such as GSNOR (Frungillo et al., 2014), NADPH oxidase (Yun et al., 2011), catalase (Ortega-Galisteo et al., 2012), and peroxiredoxinII E (Romero-Puertas et al., 2007) and II F (Camejo et al., 2015). *S*-nitrosylation appears to be critical to GSNO and $ONOO^-$ homeostasis as this NO-PTM inhibits GSNOR and PrxII E activities (Romero-Puertas et al., 2007; Frungillo et al., 2014) that decompose GSNO and $ONOO^-$, respectively. Consequently, during a nitro-oxidative stress the inactivation of these enzymes by *S*-nitrosylation could favor the accumulation of these NO-derived molecules that in turn may increase the effects of the generated stress.

A connection has also been observed between NO and ROS pathway under different physiological and stress conditions (Corpas et al., 2011; Groβ et al., 2013; Procházková et al., 2014). Furthermore, all components of Asa-GSH cycle have been reported to be *S*-nitrosylated (Lin et al., 2012; Tanou et al., 2012) with a different effect on protein activity (Kato et al., 2013; Begara-Morales et al., 2014, 2015).

Dehydroascorbate reductase has been identified as *S*-nitrosylation target at Cys20 under no-stress conditions in *Arabidopsis*, and this Cys20 is not over-nitrosylated under salinity or cold stress (Fares et al., 2011; Puyaubert et al., 2014). Recently, it has been reported that *S*-nitrosylation at Cys20 and Cys147 negatively regulates the enzymatic activity of DHAR in potato plants (Kato et al., 2013). Furthermore, peroxisomal recombinant pea MDAR, which has only two cysteines (Cys197 and Cys68) is also inhibited by *S*-nitrosylation (Begara-Morales et al., 2015). The authors suggest using *in silico* and evolutionary analysis that Cys68 could be the most reliable residue responsible for the loss of activity following GSNO treatment. However, future experiments such as site-directed mutagenesis and/or mass spectrometry are needed to verify this postulation. In any case, it is clear that peroxisomal pea MDAR is *S*-nitrosylated by GSNO, as corroborated by the biotin-switch method, and as result the protein activity is inhibited

(Begara-Morales et al., 2015). The inhibition of DHAR and MDAR by *S*-nitrosylation (**Figure 1**) could compromise ascorbate regeneration and therefore the functioning of the cycle. Notably, in the same work it is shown that chloroplastic and cytosolic pea GR are also *S*-nitrosylated by GSNO. However, this modification does not significantly affect protein activity, as happens after tyrosine nitration (see above). In mammal cells GSNO treatment for 1h does not affect GR, although an inhibitory effect is produced after longer exposures to GSNO (Beltrán et al., 2000). In addition, human GR is inhibited by GSNO as consequence of *S*-nitrosylation of two catalytic Cys, Cys63 and/or Cys58 (Becker et al., 1995; Francescutti et al., 1996). These results suggest a different regulation of pea and mammalian GR since that pea GR activity could be unaffected by any NO-PTMs under a nitro-oxidative stress situation (Begara-Morales et al., 2015) in an attempt to maintain GSH levels and consequently the cellular redox state.

Regarding the regulation of Asa-GSH cycle by *S*-nitrosylation, the best characterized enzyme is APX. APX *S*-nitrosylation could have an essential role in physiological and stress conditions via regulation of APX activity (Correa-Aragunde et al., 2013; de Pinto et al., 2013; Begara-Morales et al., 2014), highlighting that APX can constitute a critical interface in the relationship between NO and H_2O_2 metabolism (Lindermayr and Durner, 2015). It has been suggested that *Arabidopsis* APX *S*-nitrosylation/denitrosylation mediated by auxins could be involved in the determination of root architecture (Correa-Aragunde et al., 2013, 2015). In this situation, APX1 is *S*-nitrosylated *in vivo* and auxins-mediated denitrosylation decreased the protein activity, an effect corroborated by the treatment of APX1 recombinant protein with CysNO (Correa-Aragunde et al., 2013). In contrast, de Pinto et al. (2013) reported that APX *S*-nitrosylation mediated by GSNO inhibits protein activity in tobacco plants and that this change could be related to programmed cell death (PCD). By *in silico* analysis, in the former study is postulated that the increase in APX activity is consequence of *S*-nitrosylation at Cys168, whereas in the latter it is suggested that the inactivation is due to *S*-nitrosylation at Cys32. However, Clark et al. (2000) reported that the inactivation of tobacco APX activity by GSNO could be due to the formation of an iron-nitrosyl complex between NO and the heme group's iron atom. This implies that, Cys168, which is located near heme group, could be the responsible for APX activity inactivation, and not Cys32. In this sense, further experimental data (e.g., site-directed mutagenesis) could be needed to confirm what Cys is(are) involved in the (de)activation of the protein activity.

Another study described an increase in *S*-nitrosylation of pea APX as a protective mechanism in response to salinity stress (Begara-Morales et al., 2014). In this case, the cytosolic pea APX activity is stimulated by *S*-nitrosylation *in vitro* and *in vivo*. The advantage of this work is that the sequence of pea APX contains only one Cys32, making this residue the only candidate to be *S*-nitrosylated and responsible for increasing APX activity after *S*-nitrosylation. This finding has been recently corroborated by Yang et al. (2015), who showed using proteomic and mutagenesis approaches that *S*-nitrosylation at Cys32 positively regulates APX1 activity in *Arabidopsis*. In addition, they demonstrated that *S*-nitrosylation of Cys32 plays an essential role in plant response to oxidative stress and in plant immunity. As result, *S*-nitrosylation of Cys32 appears to be responsible for increasing activity of APX (**Figure 1**).

CONCLUSION AND FUTURE PERSPECTIVES

Nitric oxide and H_2O_2 are essential signaling molecules involved in physiological processes and plant response to unfavorable conditions. These molecules share signaling pathways, so that it is not surprising to find cross-talk by which one pathway can control the function of the other. In this regard, key control points of ROS metabolism by NO are the PTMs of catalase, SODs, peroxiredoxins, and enzymes of the Asa-GSH cycle. Recent findings indicate that the antioxidant capacity of Asa-GSH cycle could be compromised under stress situations that generate nitro-oxidative stress, due to the inactivation of APX and MDAR activities by tyrosine nitration (**Figure 1**). However, APX activity is increased by *S*-nitrosylation while GR is not affected by these NO-PTMs, suggesting that GR tries to maintain GSH regeneration and therefore the cellular redox state in order to sustain the Asa-GSH cycle's resistance to nitro-oxidative cell conditions. It bears noting that APX is under dual regulation by tyrosine nitration and *S*-nitrosylation, which are two different oxidative states related to nitro-oxidative stress. In this sense, future research should delve into the regulation of Asa-GSH cycle according to the oxidative stress generated and affected cell compartments.

AUTHOR CONTRIBUTIONS

The experiments were conceived and designed by: JB, FC, and JB-M. The experiments were performed by: JB-M, BS-C, MC, RV, CM-P, and MP. The data were analyzed by: JB, FC, and JB-M. The paper was written by: JB-M and JB.

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Nitric Oxide Level Is Self-Regulating and Also Regulates Its ROS Partners

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INTRODUCTION

Nitric oxide (NO) is a free radical recognized as an omnipresent inter- and intra-cellular signaling molecule involved in the regulation of an extraordinary range of diverse cellular functions in plants (Besson-Bard et al., 2008). All NO derivatives are called reactive nitrogen species (RNS) which include more favorable structures such as nitrosonium cation (NO^+) and nitroxyl radical (NO^-) as a result of NO gaining or losing electrons, and the products of the reaction between NO and its close partners, reactive oxygen species (ROS), such as peroxynitrite ($ONOO^-$), and the NO_x compounds (NO_2, N_2O_3, and N_2O_4; Bellin et al., 2013). Although it is known that NO may regulate gene transcription and activate secondary messengers, the ways in which NO functions are still mostly unidentified (Besson-Bard et al., 2008; Palmieri et al., 2008; Gaupels et al., 2011). In the last decade, however, it has been shown that NO is also able to control different biological processes in plants by directly modifying proteins through covalent post-translational modifications (PTMs) giving rise to nitration, nitrosylation or *S*-nitrosylation (Romero-Puertas et al., 2013). *S*-nitrosylation is the covalent binding of a NO group to a cysteine residue and probably the better known NO-dependent PTM as more than 1000 proteins have been shown as targets of *S*-nitrosylation (Kovacs and Lindermayr, 2013), although the functional effect of this modification has only been analyzed in around 2% of these proteins (Astier et al., 2012; Kovacs and Lindermayr, 2013; Romero-Puertas et al., 2013). Nitration, in which 3-nitrotyrosine is produced after a nitrite group is added to the ortho-position of Tyr residues, is another NO-dependent PTM, although so far analyzed to a lesser degree than *S*-nitrosylation (N-Tyr; Vandelle and Delledonne, 2011). These PTMs are able to modify the activity, location, aggregation, or even stability of proteins (de Pinto et al., 2013; Gibbs et al., 2014; Albertos et al., 2015). We should bear in mind that NO function depends on the rate and location of its production and that NO level will determine a cytotoxic or stimulating effect (Beligni and Lamattina, 2001; Serrano et al., 2012). Thus, a precise control of NO level by switching the NO signaling off or not seems to be a crucial event for plant survival, and it appears that plants have developed many strategies to achieve it.

Reactive oxygen species (ROS) comprises oxygen derivatives species produced by the reduction of oxygen, such as superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals (·OH), peroxyl radicals (ROO·), and alkoxyl radicals (RO·) and also some non-radical compounds such as hydrogen peroxide (H_2O_2), the singlet oxygen ($^1O_2$), ozone (O_3), and hypochlorous acid ($HOCl^-$) (Halliwell and Gutteridge, 2007). Although research on ROS, which have strong oxidizing potential, initially focused on cytotoxicity, in recent years, it has become clear that they can also function as signaling molecules in most cellular processes (Baxter et al., 2014). Thus, plants have developed a means of utilizing lower concentrations of ROS as signaling molecules under certain physiological and stress conditions (Petrov and Van Breusegem, 2012). Genetic and pharmacological techniques have demonstrated that different ROS species can affect nuclear gene expression by responding to a variety of environmental stimuli (Sandalio and Romero-Puertas, 2015). However, a finely tuned

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balance between ROS scavenging and ROS production is necessary to determine their level and impact as damaging and signaling molecules (de Pinto et al., 2012; Baxter et al., 2014).

NITRIC OXIDE LEVELS ARE SELF-REGULATING

NO is generated in higher plants through a variety of mechanisms and both, oxidative (arginine or hydroxylamine-dependent) and reductive (nitrate-dependent) pathways have been described (Fröhlich and Durner, 2011; Gupta et al., 2011a) nitrate reductase (NR) being the best known pathway for NO production in plants (Rockel et al., 2002). NR is capable of reducing nitrite to NO depending on nitrite accumulation and pH levels. Moreover, NO can react reversibly with glutahione (GSH) producing GSNO, a reservoir of NO (Liu et al., 2001; Sakamoto et al., 2002). GSNO is metabolized by GSNO reductase (GSNOR) which controls NO and nitrosothiol levels, being a key enzyme in most NO-regulated processes, such as pathogen defense, root development, and nitrogen assimilation (Feechan et al., 2005; Rustérucci et al., 2007; Frungillo et al., 2014). It has recently been shown that GSNO inhibits nitrate uptake and its reduction to nitrite which would prevent NR-dependent NO production (**Figure 1**; Frungillo et al., 2014). Additionally, CO_2 elevation distinctly increased *S*-nitrosylated NR levels in plants grown under high-nitrate conditions, along with a significant decrease in NR activity similarly to that which ocurrs with chilling treatment (Cheng et al., 2015; Du et al., 2015). These results suggest that *S*-nitrosylation of NR may decrease NR activity. Interestingly, NR regulation in response to high CO_2 levels is nitric oxyde synthase-like (NOS_l)-dependent (Du et al., 2015) pointing to a regulation between the different NO-production pathways. Moreover, NO also could activate NR activity under a relative low-nitrate concentration through the interaction with the haem and molybdenum centers in NR, which enhances electron transfer during nitrate reduction (Du et al., 2008). To complete the cycle, it has been also demonstrated that NO inhibits GSNOR1 through *S*-nitrosylation avoiding at the same time GSNO degradation and regulating plant nitrosothiol levels (**Figure 1**; Frungillo et al., 2014). Thus, (S)NO feedback regulates nitrogen flux through nitrite assimilation pathways and controls its bioavailability by modulating its own consumption (**Figure 1**; Frungillo et al., 2014). In the context of hypersensitive response, NO is also able to regulate the level of its own radicals, such as $ONOO^-$ through *S*-nitrosylation of Arabidopsis peroxiredoxin II E (PrxII E) that inhibits its H_2O_2-reducing and peroxinitrite-detoxifying activities (Romero-Puertas et al., 2007).

In addition, non-symbiotic haemoglobins (nsHbs) from different species have been shown to metabolize NO, which are able to move to a solution producing nitrate (Perazzolli et al., 2006; Gupta et al., 2011b). The expression of nsHb1 is induced under low oxygen stress in different plant species when an increase in NO due to NR is assumed to occur (Gupta et al., 2011a) and *Arabidopsis thaliana* nonsymbiotic hemoglobin (AHb1) scavenges NO through production of S-nitrosohemoglobin under hypoxic stress (Perazzolli et al., 2004).

NITRIC OXIDE REGULATES REACTIVE OXYGEN SPECIES

Besides its signaling functions ROS can act as oxidizing agents on proteins, lipids and nucleic acids modifying the activity or function of these molecules and hence, the steady-state levels of ROS must be strongly regulated by scavenging systems including enzymatic and non-enzymatic antioxidants, such as superoxide dismutases (SOD) that are involved in removing superoxide radicals, catalase (CAT), and the ascorbate-glutathione cycle (ASC-GSH) made up of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), ascorbate (ASC), and glutathione (GSH; Jimenez et al., 1997; Romero-Puertas et al., 2006) involved in maintaining H_2O_2 levels under control.

NO has a half-life of only a few seconds and, once produced, interacts rapidly with ROS, giving rise to a number of RNS, such as nitrogen dioxide (NO_2), which degrade to nitrite (a precursor to NO) and nitrate in aqueous solutions (**Figure 1**; Neill et al., 2008), $ONOO^-$ after reaction with $O_2^{\cdot-}$ radical and other NO_x species. Thus, the function of NO is very much linked to ROS and the first evidence of NO and ROS crosstalk was shown during hypersensitive response (HR) in which NO and H_2O_2 cooperates to trigger hypersensitive cell death and an appropriate balance between ROS and NO production is required (Delledonne et al., 2001). Actually, during the HR, SOD accelerates $O_2^{\cdot-}$ dismutation to H_2O_2 to minimize the loss of NO by reaction with $O_2^{\cdot-}$ and to trigger hypersensitive cell death. Surprisingly, very recently it has been shown that when S-nitrosothiols are high during the HR, nitric oxide governs a negative feedback loop limiting the hypersensitive response by *S*-nitrosylation of the NADPH oxidase, the enzyme that produces $O_2^{\cdot-}$ radicals (**Figure 1**; Yun et al., 2011). NO is not only able to regulate $O_2^{\cdot-}$ production but also H_2O_2 as it has been shown that the *S*-nitrosylation pattern of glycolate oxidase (GOX), one of the main H_2O_2 sources in the peroxisome, changes in response to Cd (Ortega-Galisteo et al., 2012).

A number of antioxidant enzymes have been shown to be regulated by NO, meaning that NO is also able to modulate ROS levels by regulating the antioxidant system. Thus, peroxynitrite inhibits through Tyr nitration the mitochondrial manganese SOD1 (MSD1), peroxisomal copper/zinc SOD3 (CSD3), and chloroplastic iron SOD3 (Holzmeister et al., 2015). Although SODs have been identified as candidates for *S*-nitrosylation in different species, the effect of this PTM on the function of SODs has not so far been confirmed (Lindermayr et al., 2005; Tanou et al., 2009; Sehrawat et al., 2013), and the activity appears to be unaffected by GSNO in Arabidopsis recombinant proteins (Holzmeister et al., 2015). Additionally, catalase (CAT), one of the main enzymes involved in degrading H_2O_2 produced in peroxisomes, is *S*-nitrosylated and nitrated, causing both PTMs a loss of protein activity (Lozano-Juste et al., 2011; Ortega-Galisteo et al., 2012; Chaki et al., 2015). NO can react with the haem group of proteins, being CAT and APX reversibly inhibited during the resistance response, thus supporting a role for NO in regulating H_2O_2 levels in this context (Clark et al., 2000). All the enzymes of the ASC-GSH cycle also appear to

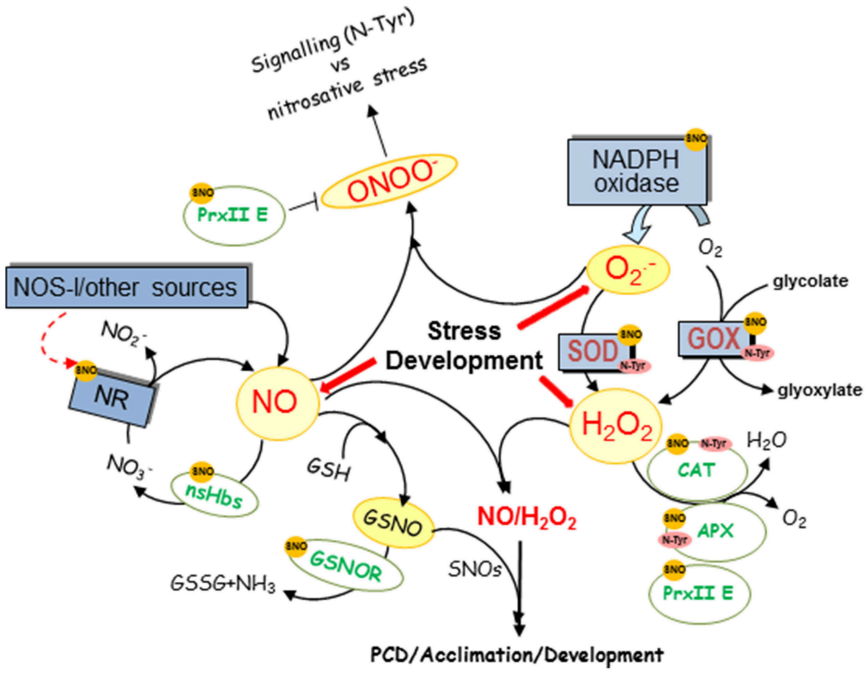

FIGURE 1 | Overview of NO and ROS level regulation by NO. NO regulates through posttranslational modifications (PTMs), NO and ROS producing and scavenging enzymes and the figure shows a diagram of the main targets of S-nitrosylation (SNO), nitrosylation (Haem-NO), or nitration (N-Tyr) described in plants. CAT, catalase; GSH, glutathione; GOX, glycolate oxidase; GSNO, nitrosoglutathione, GSNOR, GSNO reductase; nsHbs, non-symbiotic hemoglobins; NOS-l, activity that resembles NO production as catalyzed by the animal enzyme NOS; NiNOR, plasma membrane-bound NiNOR; NR, nitrate reductase; XOR, xanthine oxidoreductase.

be regulated by NO through *S*-nitrosylation and/or nitration, APX being the one directly involved in H_2O_2 detoxification and the most studied (Correa-Aragunde et al., 2015). Whilst APX activity has been shown to be inhibited by nitration (Begara-Morales et al., 2014) differing results have been found regarding the effect of *S*-nitrosylation on APX. Thus, *S*-nitrosylation of APX avoids carbonylation of the protein in seeds of *A. toxicaria* (Bai et al., 2011), increases its activity in salt stressed pea plants (Begara-Morales et al., 2014), enhances its activity by increasing resistance to oxidative stress and playing an important role in regulating immune responses (Yang et al., 2015), and under heat shock and H_2O_2 conditions during PCD in tobacco BY2 cells an inhibition of enzyme activity by *S*-nitrosylation, ubiquitination and degradation of APX has been described (de Pinto et al., 2013). It has also been shown that APX1 denitrosylation causes the partial inhibition of APX1 activity during root development (Correa-Aragunde et al., 2013). From these results it appears that the level of stress conditions (Correa-Aragunde et al., 2015), specific environment or possibly the species involved may affect the effect of *S*-nitrosylation on APX.

CONCLUSIONS

In most NO regulated processes, plant response is not activated by NO alone but is the result of a network of connections between different signaling molecules and pathways, especially the ROS-dependent ones. In many cases, the level of NO, ROS, and their balance will determine cell fate and recent research has uncovered several key NO-dependent PTMs showing that NO is able to self-regulate and also regulates ROS levels, allowing the plant to fine-tune specific responses to different stimuli. However the identification of additional components of NO regulated networks, especially those that are involved in ROS metabolism, is required to fully understand this process. Capturing the whole scene may not be an easy task however, and some questions remain to be answered. Although NO has been shown to be able to modify proteins through different PTMs, it is still unclear what triggers a specific PTM in response to a specific signal and how crosstalk between different PTMs in the same protein is regulated. NO-dependent PTMs have also been shown to affect the stability of certain proteins. However, as has been demonstrated in relation to many hormonal pathways, we still do not know whether ubiquitin-mediated protein degradation is a central regulatory mechanism in NO-signaling. Further study is required to clarify this issue.

AUTHOR CONTRIBUTIONS

MP wrote the article and LS discussed and commented on the manuscript.

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MINI REVIEW
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In Search of Enzymes with a Role in 3′, 5′-Cyclic Guanosine Monophosphate Metabolism in Plants

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In plants, nitric oxide (NO)-mediated 3′, 5′-cyclic guanosine monophosphate (cGMP) synthesis plays an important role during pathogenic stress response, stomata closure upon osmotic stress, the development of adventitious roots and transcript regulation. The NO-cGMP dependent pathway is well characterized in mammals. The binding of NO to soluble guanylate cyclase enzymes (GCs) initiates the synthesis of cGMP from guanosine triphosphate. The produced cGMP alters various cellular responses, such as the function of protein kinase activity, cyclic nucleotide gated ion channels and cGMP-regulated phosphodiesterases. The signal generated by the second messenger is terminated by 3′, 5′-cyclic nucleotide phosphodiesterase (PDEs) enzymes that hydrolyze cGMP to a non-cyclic 5′-guanosine monophosphate. To date, no homologues of mammalian cGMP-synthesizing and degrading enzymes have been found in higher plants. In the last decade, six receptor proteins from *Arabidopsis thaliana* have been reported to have guanylate cyclase activity *in vitro*. Of the six receptors, one was shown to be a NO dependent guanylate cyclase enzyme (NOGC1). However, the role of these proteins *in planta* remains to be elucidated. Enzymes involved in the degradation of cGMP remain elusive, albeit, PDE activity has been detected in crude protein extracts from various plants. Additionally, several research groups have partially purified and characterized PDE enzymatic activity from crude protein extracts. In this review, we focus on presenting advances toward the identification of enzymes involved in the cGMP metabolism pathway in higher plants.

Keywords: nitric oxide, cGMP, phosphodiesterases, plants, guanylate cyclase, signaling

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INTRODUCTION

The second messenger 3′, 5′-cyclic guanosine monophosphate (cGMP) discovered in the 1960s is found in both prokaryotes and eukaryotes (Ashman et al., 1963; Eckstein, 1988; Müller, 1997; Cadoret et al., 2005). The molecule cGMP is synthesized from guanosine triphosphate (GTP) by guanylate cyclase enzymes (GCs) and is involved in various cellular responses, such as protein kinase activity, cyclic nucleotide gated ion channels and cGMP regulated cyclic nucleotide phosphodiesterases (Denninger and Marletta, 1999; Gomelsky and Galperin, 2013). The signal generated by the second messenger is halted by 3′, 5′-cyclic nucleotide phosphodiesterase (PDE) enzymes (Hoyer et al., 1994; Richter, 2002; Maurice et al., 2014). In mammals, the metabolism and physiological role of cGMP is

well characterized, this cyclic nucleotide is important in olfactory signaling (Pietrobon et al., 2011), visual adaptation (Vielma et al., 2011) and vasodilation (Thoonen et al., 2013). In contrast, the role of cGMP in plants is not well understood.

Cyclic nucleotide monophosphates (cNMP) levels including cGMP are lower in plants relative to other eukaryotes and as a result the detection and quantification of cNMP was challenging for several decades (Newton and Smith, 2004). However, with the development of sensitive methods, it is now possible to measure and quantify cNMP *in planta*. The methods include; mass spectrometry based measurements (Newton and Smith, 2004), radiolabeled (Steiner et al., 1972) and antibody (Lomovatskaya et al., 2011) based immunoassays. In addition, non-invasive techniques have been developed that allow the detection of endogenous cytoplasmic cGMP levels *in vivo*, these include a fluorescent cGMP biosensor called FlincG (Isner and Maathuis, 2011) and a cGMP responsive promoter fused to a luciferase reporter gene (Wheeler et al., 2013). Consequently, in the past decade, several research groups have shown a positive correlation between the accumulation of cGMP *in planta* and various developmental processes as well as the response to abiotic and pathogenic stress (**Figure 1**; Durner et al., 1998; Pagnussat et al., 2003b; Szmidt-Jaworska et al., 2004; Maathuis, 2006; Suita et al., 2009; Teng et al., 2010; Joudoi et al., 2013; Li et al., 2014; Nan et al., 2014). For instance, Durner et al. (1998) were the first group to show a correlation between nitric oxide (NO) dependent cGMP synthesis and pathogen defense response in *Nicotiana tabacum*. The elevated levels of cGMP in turn activated the expression of a pathogenic marker, phenylalanine ammonia lyase (PAL). In the subsequent years, several research groups have documented that both NO-dependent and NO-independent cGMP signaling pathways are important in the activation of defense responses during biotic stress (Klessig et al., 2000; Ma et al., 2007; Meier et al., 2009; Pasqualini et al., 2009; Qi et al., 2010; Ma et al., 2013). Additionally, NO-cGMP dependent signaling pathway has been reported to be involved in the development of adventitious roots (Pagnussat et al., 2003a,b; Xuan et al., 2012), stomata closure during abiotic and biotic stress (Neill et al., 2008; Joudoi et al., 2013), protein phosphorylation (Isner et al., 2012; Marondedze et al., 2015) and transcription regulation (Suita et al., 2009).

Collectively, research in the last two decades suggests that cGMP is an important second messenger in plants, albeit, the metabolism of cGMP in plants is not well understood. In this review, we focus on advances toward the identification of enzymes involved in the metabolism of cGMP in plants.

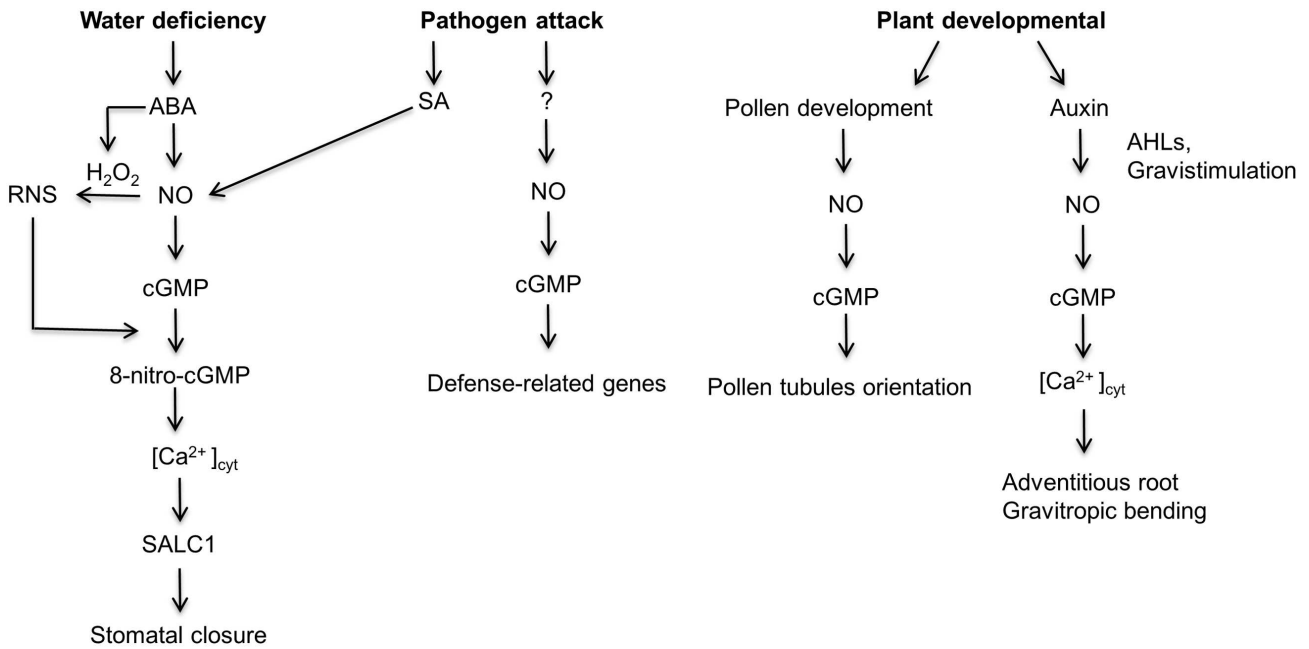

FIGURE 1 | A schematic depiction of the nitric oxide-induced cGMP signaling pathway in developmental, abiotic and biotic stress processes. During water stress, an increase in the hormone abscisic acid (ABA) activates the synthesis of nitric oxide (NO). Subsequently, NO stimulates the NO-dependent guanylate cyclase to produce cGMP (Dubovskaya et al., 2011; Joudoi et al., 2013). Concurrently, ABA also activates the production of H_2O_2 which reacts with NO to produces reactive nitrogen species (RNS; Joudoi et al., 2013). A reaction between the RNS and cGMP produce 8-Nitro-cGMP which in turn activates the accumulation of cytoplasmic calcium, $[Ca2^{+}]_{cyt}$ and the SLOW ANION CHANNEL 1 (SLAC1) which results in stomatal closure. Similarly, during a pathogenic attack, plants close their stomata; however, the NO-cGMP pathway is initiated by the hormone salicylic acid (SA; Hao et al., 2010). Furthermore, during a pathogenic attack, NO-cGMP signaling cascade activates the transcription of the pathogenic marker, PAL in an SA-independent manner (Durner et al., 1998). Furthermore, NO-cGMP signaling pathway is important during pollen tubule development. NO-cGMP signaling pathway is also involved in adventitious root formation stimulated by exogenous and endogenous chemicals, for example, *N*-Acyl-homoserine-lactones (AHLs) produced by gram negative rizobacteria. AHLs promote polar auxin transport which activates the NO-cGMP dependent signaling cascade leading to the development of adventitious root formation (Pagnussat et al., 2003a; Lanteri et al., 2006; Bai et al., 2012). Similarly, gravitropism bending requires the auxin induced NO-cGMP signaling pathway (Hu et al., 2005).

NITRIC OXIDE DEPENDENT GUANYLATE CYCLASE ENZYMES IN PLANTS

In the plant kingdom, genes coding for nucleotide cyclase (NCs) enzymes have been identified in lower plants from the division of Chlorophyta. For instance *Chlamydomonas reinhardtii* contains more than 90 NCs enzymes (Meier et al., 2007; Marondedze et al., 2016). Among these annotated NCs, NO-induced GC enzymes homologous to those found in mammalian species have been identified (Winger et al., 2008; de Montaigu et al., 2010). In higher plants, protein sequences with high homology to known GCs have not been identified. However, motif searches based on functionally assigned amino acid residues within the catalytic center has resulted in the identification of several proteins that have been shown to have guanylate cyclase activity *in vitro*. These include; phytosulfokine (PSK) receptor, AtPepR1, *Arabidopsis thaliana* guanylate cyclase 1 (AtGC1), brassinosteroid receptor (AtBRII), plant natriuretic peptide receptor (AtPNP-R1) and wall associated kinase-like 10 (AtWAKL10: Ludidi and Gehring, 2003; Kwezi et al., 2007; Meier et al., 2010; Qi et al., 2010; Kwezi et al., 2011; Turek and Gehring, 2016). These receptors synthesize cGMP from GTP independent of NO.

In an effort to identify NO-dependent GCs in plants, Mulaudzi et al. (2011) searched the *Arabidopsis thaliana* sequence database for the conserved residues within the catalytic center as well as the heme-nitric oxide and oxygen binding domain (H-NOX), a domain required for the binding of NO in GCs. The authors found one protein annotated as a Flavin-dependent monooxygenase (At1g62580) that contained both the H-NOX motif and the conserved amino acid residues within the catalytic motif. The enzyme was termed NO dependent guanylate cyclase 1 (NOGC1). Interestingly, stomata closure did not occur in *nogc1* T-DNA knockout mutants lines when treated with an NO donor, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene, compared to wild type plants (NOC5; Joudoi et al., 2013). These exciting results suggest that NOGC1 is involved in the NO-cGMP signaling pathway in regard to stomatal closure. Furthermore, *in vitro* assays confirmed that the recombinant protein NOGC1 has a higher affinity for NO than oxygen (Mulaudzi et al., 2011). However, although NOGC1 recombinant protein is able to synthesize cGMP in an NO dependent manner, cGMP is produced in extremely low amounts (400–450 fmol/μg in 20 min) relative to GCs found in mammals, for example the recombinant sGC from human (940 pmol/min/μg; Kosarikov et al., 2001). It could be possible that additional unknown cofactors are required for the optimal function of this enzyme and therefore, the assay conditions *in vitro* are not ideal. Indeed this is true for the phytosulfokine receptor 1 (PSKR1) which showed an increase in GC activity in the presence of calcium (Muleya et al., 2014). Furthermore, there are additional factors that affect the production of active recombinant proteins; these are discussed in detail by Bernaudat et al. (2011) and references therein. As suggested by Wong and Gehring (2013), it is important that further studies are carried out *in vivo*. For example, the synthesis of cGMP by NOGC1 could be studied in plants containing the cGMP biosensor FlincG (Isner and Maathuis, 2011). The advantage of this system is that the detection of cGMP is non-invasive; therefore, continuous real-time changes in cGMP could be studied. A further advantage is that cytosolic cGMP levels can be detected in specific organelles, thus, allowing high resolution measurements.

In contrast to the recent developments in the identification of NO induced GCs in plants, the publications concerning the identification of plant specific 3′, 5′-cyclic nucleotide phosphodiesterase enzymes (PDEs) responsible for the degradation of cGMP stopped in 2001. It is peculiar that there has been no research output in regard to the identification of PDEs from plants in the last 16 years. The next section provides a summary on the efforts in the identification of PDEs and the comparison of their physical and chemical properties to the PDEs found in prokaryotes, lower and higher eukaryotes.

3′, 5′-CYCLIC UANOSINE MONOPHOSPHATE PHOSPHODIESTERASES FOUND IN PROKARYOTIC AND EUKARYOTIC ORGANISMS

3′, 5′-cyclic nucleotide phosphodiesterase enzymes (PDEs) degrade cGMP and the other second messenger, 3′, 5′-cyclic adenosine monophosphate (cAMP). The PDE superfamily is classified into three groups (Class I, Class II, and Class III) based on sequence and structural similarities (**Figure 2**). Class I PDEs are found in higher and lower eukaryotic organisms including all mammalian species and numerous species belonging to the Amoebozoa and Fungi kingdoms (Sass et al., 1986; Michaeli et al., 1993; Rosman et al., 1997; Thomason et al., 1998; Fawcett et al., 2000; Fujishige et al., 2000; Hetman et al., 2000; Kuwayama et al., 2001; Wang et al., 2001; Lee et al., 2002; Richter, 2002; Huai et al., 2003; Jung and Stateva, 2003; Wang et al., 2003; Zhang et al., 2005; Bader et al., 2006; Corbin et al., 2006; Muradov et al., 2010; DeNinno et al., 2011; Barnes, 2013; Zhu et al., 2013; Du et al., 2014; Maurice et al., 2014). Class II PDEs are found in lower eukaryotes and bacteria (Van Haastert et al., 1983; Van Haastert and Van Lookeren Campagne, 1984; Lacombe et al., 1986; Dunlap and Callahan, 1993; Hoyer et al., 1994; Imamura et al., 1996; Degerman et al., 1997; Bosgraaf et al., 2002; Bader et al., 2007; Tian et al., 2014). Finally, members of class III belong exclusively to PDEs isolated from prokaryotes (Imamura et al., 1996; Richter, 2002; Shenoy et al., 2005; Zheng et al., 2013). Although Class I, II, and III PDEs have different sequence and structure homology, they use similar mechanisms in the hydrolysis of cNMP by cleaving the ester bond at carbon 3′ to produce a non-cyclic 5′ NMP. These enzymes belong to a superfamily called binuclear metallohydrolases (Mitić et al., 2006).

Partially Purified PDEs from Plants with Promiscuous Enzyme Activity

In the plant kingdom, lower plants from the division of Chlorophyta contain genes coding for classic Class I PDEs within their genome. As shown by our phylogenetic tree analysis,

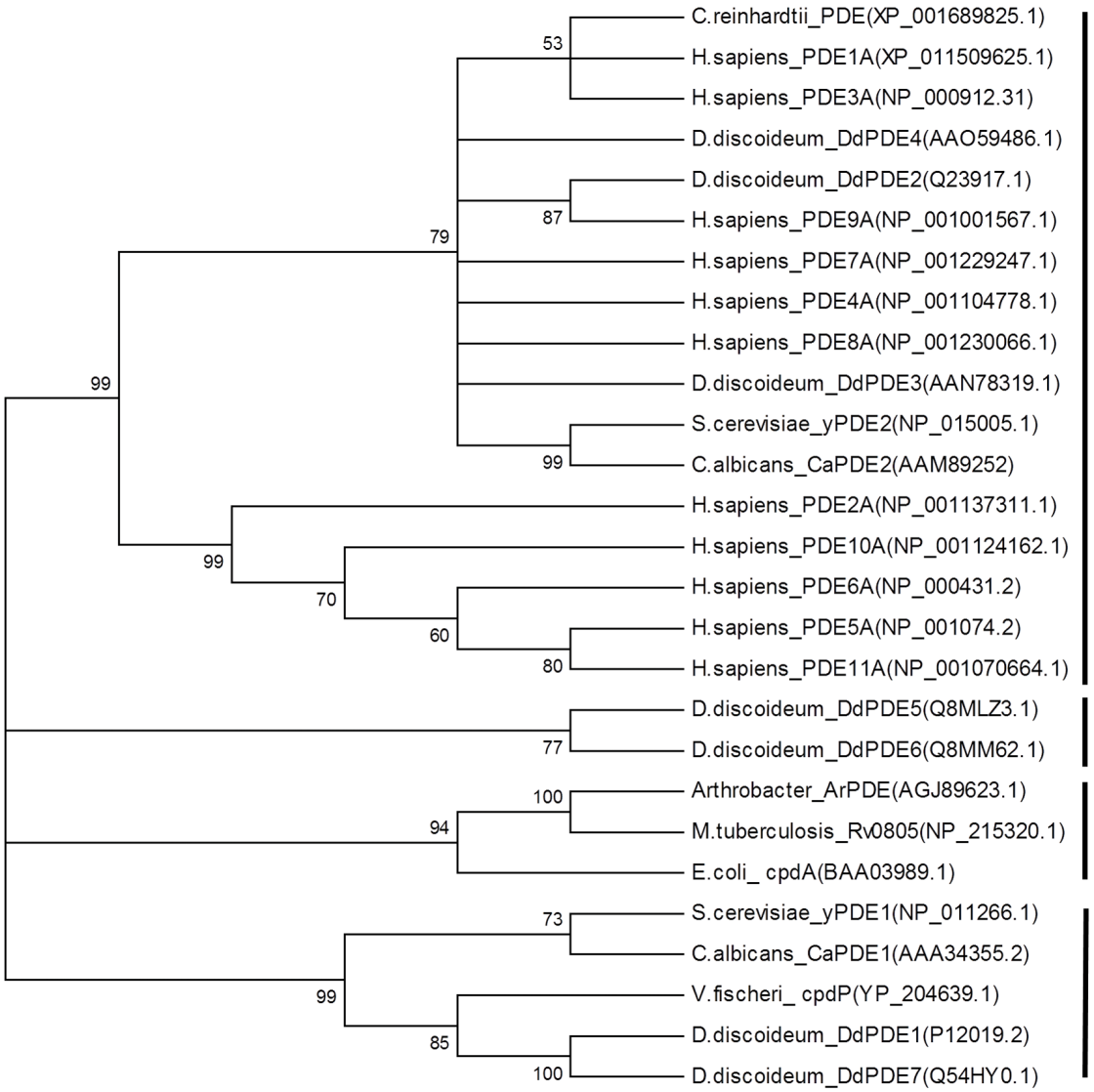

FIGURE 2 | Phylogenetic analysis of biochemically characterized 3′, 5′-cyclic nucleotide monophosphate phosphodiesterase (PDEs) found in prokaryotes, lower and higher eukaryotes. The protein sequences of characterized PDEs from Class I, II, and III were retrieved from GenBank. The PDEs belong to *Chlamydomonas reinhardtii* (algae), *Homo sapiens* (human), *Dictyostelium discoideum* (Slime mold), *Saccharomyces cerevisiae* (fungi), *Candida Albicans* (fungi), Arthrobacter (proteobacteria), *Escherichia coli* (proteobacteria), *Mycobacterium tuberculosis* and *Vibrio fischeri*. A multiple alignment with the retrieved PDEs was performed in a protein alignment program, Muscle (Edgar, 2004). The phylogenetic tree was constructed in MEGA6 (Tamura et al., 2013) using the Neighbor-joining statistical method with a bootstrap replication number of 1000. Members of the Class I PDEs are found in lower and higher eukaryotes and Class II PDEs are found in lower eukaryotes as well as bacteria. Finally, Class III PDEs are found exclusively in bacteria.

a PDE from *Chlamydomonas reinhardtii* is closely related to mammalian Class I PDE 1A and 3A (**Figure 2**). These findings complement studies by Fischer and Amrhein (1974) who showed that a partially purified protein from *C. reinhardtii* exhibited typical Class I enzyme activity during the hydrolysis of cAMP and cGMP. This enzyme was also able to hydrolyze 3′, 5′-cyclic cytosine monophosphate (cCMP) and the hydrolysis was threefold and sixfold higher than cAMP and cGMP, respectively. These findings are compatible with recent data that shows that mammalian PDEs exhibit promiscuous substrate specificity toward other 3′, 5′-cyclic nucleotides than previously reported, for example, cyclic uridine monophosphate (cUMP), cyclic thymidine monophosphate (cTMP) and cyclic inosine monophosphate (cIMP; Reinecke et al., 2011).

In higher plants such as angiosperms, PDEs have not been identified; however, PDE activity has been detected in crude protein extracts from various plants. In an effort to purify PDEs, crude extract from *Solanum tuberosum* (Ashton and Polya, 1975; Zan-Kowalczewsk et al., 1984), *Pisum sativum* (Lin and Varner, 1972), *Nicotiana tabacum* (Shinshi et al., 1976; Matsuzaki and Hashimoto, 1981) and *Solanum lycopersicum* (Abel et al., 2000) were subjected to sequential purification steps that

included ammonium sulfate precipitation, ion chromatography and gel filtration chromatography. Molecular weights of protein fractions with PDE activity were determined by gel filtration with markers of known molecular weight. Proteins capable of hydrolyzing cNMP were present as monomeric as well as tetrameric protein complexes. The molecular weight values for the monomer and tetramer PDEs range from 60 000–75 000 and 270 000–350 000, respectively and they all possess similar enzymatic activity properties. In other plants, the partially purified PDEs appear in fractions at lower molecular weights than the proposed size of the monomeric form, 60 000–75 000. In *Spinacea oleracea*, the sizes of partially purified PDEs with estimated sizes of 50 and 37 KDa were reported (Brown et al., 1979). All partially purified PDEs mentioned were from crude cytosolic (soluble) proteins. However, Vandepeute et al. (1973) report the partial purification of PDE from both soluble proteins and particulate (membrane) proteins, suggesting that plants could possess both cytosolic and membrane bound PDEs.

In contrast to Class I, II, and III PDEs, all mentioned PDEs above hydrolyze 2′, 3′ and 3′, 5′-cNMPs with most of them showing a higher affinity toward the hydrolysis of 2′, 3′ cNMP than 3′, 5′-cNMP (Supplementary Table S1; Lin and Varner, 1972; Ashton and Polya, 1975; Brown et al., 1979; Endress, 1979; Abel et al., 2000). It is probable that unlike other organisms, plants contain PDEs with dual enzymatic function. This claim is supported by the finding from a partially purified PDE (70 kDa) from *S. lycopersicum* that was shown to be induced and excreted from suspension-cultured cells during inorganic phosphate deficiency (Abel et al., 2000). It was proposed that together with ribonuclease I and acid phosphatases, plant PDEs are expressed and secreted to degrade extracellular RNA, releasing inorganic phosphate that is in turn transported into the cells via Pi transporters. The authors speculate that due to its higher affinity for 2′, 3′ cNMPs than 3′, 5′-cNMPs and because it is excreted during phosphate deficiency, the PDE identified is involved in RNA turnover rather than the degradation of 3′, 5-cNMP and thus the enzyme was named 2′, 3′-cyclic nucleotide-phosphodiesterase. An alternative suggestion is that in plants, PDEs and nucleotide pyrophosphates are part of a multi-complex structure and because they possess similar properties such as isoelectric point and molecular weight, they co-purify during the isolation process. This is a more convincing theory as other groups have reported partially purified PDEs that specifically degrade 3′, 5′-cNMP isomers; these are discussed in the following section.

Partial Purification of 3′, 5′-Cyclic Nucleotide Monophosphate Specific PDEs in Plants

To isolate protein with PDE activity from *Phaseolus vulgaris*, the authors extracted total soluble extract from the vegetative tissue and centrifuged it at 100 000 *g* (Brown et al., 1977). The pellet was retained and dissolved in 50 mM Tris–HCL buffer (pH7.4) and the following purification steps were performed; ammonium sulfate precipitation and gel filtration (Sephadex G-200). Two fractions containing PDE activity were detected with molecular weights of 340 and 76 KDa, confirming that in plants PDEs could exist in monomeric and tetrameric forms. In addition, both enzyme fractions produced a mixture of 3′ NMP and 5′ NMP after the hydrolysis of 3′, 5′-cNMPs, similar to all partially purified plant PDEs described to date. Interestingly, PDE from *P. vulgaris* was described to specifically hydrolyze 3′, 5′-cNMP and had no activity against 2′, 3-cAMP. The substrate preference is in the following order: 3′, 5′-cAMP, 3′, 5′-cGMP, 3′, 5′-cUMP and 3′, 5′-cCMP (Brown et al., 1977). Similarly, partially purified PDE from *S. oleracea* and *P. volganic* were reported to have catalytic preference for 3′, 5′-cNMP (Brown et al., 1980; Diffley et al., 2001). The PDE from *S. oleracea* hydrolyzed both 3′, 5′-cAMP and 3′, 5′-cAMP and divalent metals were not required for optimal enzyme activity. The hydrolysis of 3′, 5′-cAMP and 3′, 5′-cAMP by the partially purified PDE from *P. volganic* was activated and inhibited by Mg^{2+}/Mn^{2+} and Fe^{3+}, respectively. These results indicate that the PDE from *P. volganic* requires divalent metals similar to CLASS I PDEs found in eukaryotic organisms. However, in contrast to CLASS I PDEs, the presence of Fe^{3+} activated the hydrolysis of 3′, 5′-cCMP (Diffley et al., 2001).

The Future for Plant Specific PDE Research

It is possible to speculate that with the advances in proteomics in the last 16 years, there is a high chance that plant-specific PDEs could be identified from the sequentially fractionated samples. For example, the refined liquid chromatography coupled mass spectrometry (LC MS/MS) based proteomics analysis techniques are now capable of deciphering protein mixtures. The sensitivity of the techniques allows the identification of proteins in low abundance (Ahlf et al., 2013; Di Girolamo et al., 2013; Fukao et al., 2013). Furthermore, peptides fingerprints/proteins from the mass spectrometry analysis can be readily identified as the genomes of 49 plants species have been sequenced since 2001 (Michael and Jackson, 2013). These include the genomes of *S. tuberosum* (Xu et al., 2011), *N. tabacum* (Sierro et al., 2014), and *S. lycopersicum* (Daniell et al., 2006).

CONCLUSION

Nitric oxide-dependent cGMP production is involved in various signaling processes in plants particularly in (i) the control of stomatal aperture which is important in surviving water deficit (Neill et al., 2008; Joudoi et al., 2013) and (ii) defense response during pathogen attack (Durner et al., 1998; Ma et al., 2013). NOGC1 is the first NO-dependent GC identified in plants; however, the function of this protein remains to be elucidated *in planta*. As stated, the last publication on identifying plant specific PDEs was in 2001. Finding plant PDEs could have a substantial impact in understanding the NO-cGMP pathway and its physiological effects. As cGMP is positively correlated with plants adapting to environmental stresses, the discovery of PDEs will lead to the development of plant-specific PDE

inhibitors which would maintain intracellular levels of cGMP. In mammals, perturbation in the synthesis as well as degradation of cGMP results in various human ailments (Maurice et al., 2014). Inhibitors of mammalian PDEs are used as therapeutic agents to regulate the concentrations of intracellular cGMP and thus alleviate diseases. It is safe to postulate that plant specific PDE inhibitors could be important in improving crop performance during environmental stress and thus could have similar commercial value as the well-studied mammalian PDEs.

AUTHOR CONTRBUTIIONS

The first and corresponding author, IG initiated and wrote the review. JD proof-read the manuscript and gave intellectual input.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00576

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