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Redox homeostasis via gene families of ascorbate-glutathione pathway

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The imposition of environmental stresses on plants brings about disturbance in their metabolism thereby negatively affecting their growth and development and leading to reduction in the productivity. One of the manifestations of different abiotic and biotic stress conditions in plants is the enhanced production of reactive oxygen species (ROS) which can be hazardous to cells. Therefore, in order to protect themselves against toxic ROS, plant cells employ the anti-oxidant defense system. The ascorbate-glutathione pathway (Halliwell-Asada cycle) is an indispensible component of the ROS homeostasis mechanism of plants. This pathway entails the antioxidant metabolites: ascorbate, glutathione and NADPH along with the enzymes linking them. The ascorbate-glutathione pathway is functional in different subcellular compartments and all the enzymes of this pathway exist as multiple isoforms. The expression of different isoforms of the enzymes of ascorbate-glutathione pathway is developmentally as well as spatially regulated. Moreover, various abiotic and biotic stress conditions modulate the expression of the enzyme- isoforms differently. It is the intricate regulation of expression of different isoforms of the ascorbate-glutathione pathway enzymes that helps in the maintenance of redox balance in plants under various abiotic and biotic stress conditions. The present review provides an insight into the gene families of the ascorbate-glutathione pathway, shedding light on their role in different abiotic and biotic stress conditions as well as in the growth and development of plants.

Keywords: reactive oxygen species, abiotic stress, redox homeostasis, ascorbate-glutathione pathway, isoforms, gene families

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Introduction

When plants are exposed to various biotic and abiotic stresses, they exhibit characteristic increase in the production of reactive oxygen species (ROS) like singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) (Mittler et al., 2004). These ROS are capable of causing uncontrolled oxidation of various cellular components that can lead to oxidative damage of the cell (Asada, 1999; Dat et al., 2000). Thus, enhanced production of ROS during stress can be hazardous to cells. ROS have also been acknowledged as central players in complex signaling cascades as they act as signals for the activation of various stress-responsive and defense pathways (Knight and Knight, 2001; Mittler et al., 2011). Apart from playing important roles in stress signaling, ROS like H_2O_2 are also involved in plants' growth and developmental processes like differentiation of cellulose rich cell wall, mediation of aleuronic

cell death and stimulation of somatic embryogenesis (Neill et al., 2002; Slesak et al., 2007). Additionally, the transient accumulation of H_2O_2 following pathogen infection leads to localized programmed cell death or hypersensitive (HR) response and stimulates crosslinking of cell wall proteins, thereby preventing pathogen spread in the plant (Grant and Loake, 2000; Kuzniak and Skłodowska, 2005). Considering the ambivalent role of ROS, a delicate balance between their production and scavenging is of utmost importance for proper growth and development of plants.

Plants have an efficient anti-oxidant defense system which scavenges the excess ROS produced in the cell under different oxidative stress conditions. The anti-oxidant safe guard system in plants comprises of non-enzymatic and enzymatic components (Noctor and Foyer, 1998; Scandalios, 2005). The non-enzymatic components include the major cellular redox buffers: ascorbate (AsA) and glutathione (γ-glutamyl-cysteinyl-glycine, GSH) as well as tocopherol, flavonoids, alkaloids, and carotenoids (Arora et al., 2000; Grace and Logan, 2000; Foyer and Noctor, 2003; Gomathi and Rakkiyapan, 2011). The enzymatic components of the anti-oxidative defense system consist of a number of anti-oxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and the enzymes of ascorbate-glutathione (AsA-GSH) cycle namely, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Mittler et al., 2004; Scandalios, 2005). AsA-GSH cycle forms the main H_2O_2-detoxification system operating in cytosol, chloroplasts and mitochondria of plant cells (Noctor and Foyer, 1998; Shigeoka et al., 2002; Mittler et al., 2004). Since the discovery of the AsA-GSH cycle in the mid-1970s, the enzyme-catalyzed reactions of this pathway have been studied intensively (Noctor and Foyer, 1998; Asada, 1999; Polle, 2001). Studies with mutants and transgenic plants over- or under-expressing enzymes or metabolites of the AsA-GSH pathway have proved very well the co-relation between this pathway and stress tolerance (Gill and Tuteja, 2010). The AsA-GSH cycle not only combats oxidative stress, but also plays an important role in other plant developmental processes (Chen and Gallie, 2006; Eastmond, 2007).

Each enzyme of the AsA-GSH pathway has various subcellular isoforms, which differ from each other with respect to their spatial and temporal expression. Moreover, these isoforms are differentially regulated by different types of stresses. For example, it has been found that under salt stress, various *Oryza sativa* APX (OsAPX) isoforms are differentially regulated. While some of them are characteristically up-regulated, the others are down-regulated at the same time (Texeira et al., 2006; Yamane et al., 2010). This suggests that the expression of different isoforms of the AsA-GSH pathway enzymes is under intricate regulation. However, the mechanisms underlying the regulation of these isoforms are not completely understood. The present review provides an overview of gene families encoding AsA-GSH pathway in plants and imparts an insight into their role in conferring tolerance to various abiotic and biotic stresses. A brief discussion on the functional importance of this pathway in growth and development of plants is also provided.

The Ascorbate-Glutathione (AsA-GSH) Pathway

The AsA-GSH pathway is composed of four enzymes namely, APX, MDHAR, DHAR and GR (**Figure 1**) and two anti-oxidants, AsA and GSH. APX, which is the first enzyme of the cycle, detoxifies H_2O_2 by bringing about the peroxidation of AsA and yielding monodehydroascorbate radical (MDHA). MDHA is either directly reduced back to AsA by MDHAR or undergoes non-enzymatic disproportionation to AsA and dehydroascorbate (DHA). The next step of the cycle involves DHAR mediated

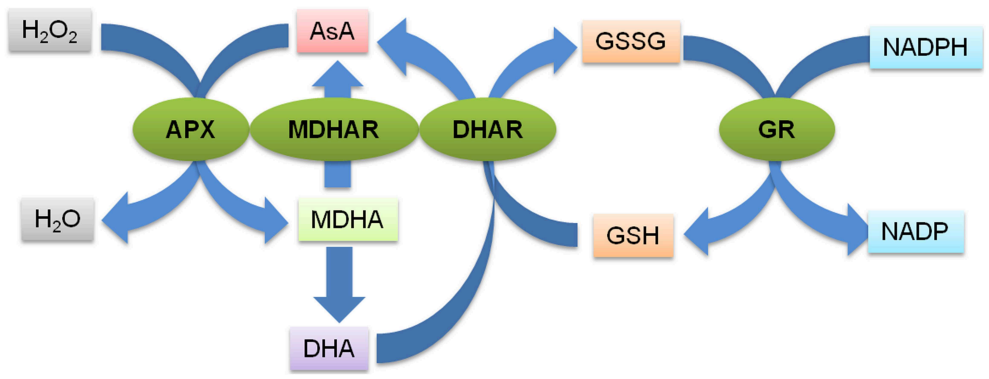

FIGURE 1 | Schematic representation of the AsA-GSH pathway. The first step of the pathway is the detoxification of H_2O_2 by APX catalyzed peroxidation of AsA which generates MDHA. MDHA is either reduced back to AsA by MDHAR or it undergoes non-enzymatic disproportionation to AsA and dehydroascorbate (DHA). The DHA molecules are reduced to AsA by DHAR using GSH as the reductant. GSH is regenerated from the oxidized glutathione dimers (GSSG) by NADPH-dependent GR. The green ovals indicate the various enzymes catalyzing the different steps of the pathways (indicated by the blue arrows). APX, Ascorbate peroxidase; MDHAR, Monodehydroascorbate reductase; DHAR, Dehydroascorbate reductase; GR, Glutathione reducatse, AsA, Ascorbic acid; GSH, Glutathione; GSSG, oxidized glutathione dimer; MDHA, Monodehydroascorbate; DHA, Dehydroascorbate.

reduction of DHA into AsA using GSH as the reductant (**Figure 1**). DHA can undergo irreversible hydrolysis to 2, 3-diketogulonic acid, if not reduced by DHAR. Thus, DHAR helps in regeneration of AsA and plays an important role in maintaining the cellular AsA pool (Gallie, 2012). Like AsA, the regeneration of GSH is also important. GSH is regenerated from the oxidized glutathione dimers (GSSG) by NADPH-dependent GR (Gill and Tuteja, 2010). The concentration of AsA and GSH varies in different subcellular compartments of the cell (**Table 1**). Both the redox buffers are known to accumulate in certain cellular compartments under different abiotic stress conditions. The compartment specific role of both the buffers under abiotic stress conditions has been discussed exhaustively in recent reviews (Foyer and Noctor, 2011; Gest et al., 2013; Zechmann, 2014), and we do not focus on this aspect in the present review. The AsA-GSH cycle not only detoxifies toxic H_2O_2 but also contributes toward the maintenance of cellular AsA and GSH pools in different compartments of the cell. Existing in all the organelles, the AsA-GSH pathway protects the cell from the toxic effects of ROS generated under a variety of abiotic and biotic stresses (Anjum et al., 2010, 2014; Gill and Tuteja, 2010) (**Figure 2**).

Ascorbate Peroxidase

APX (EC 1.11.1.11) is the first enzyme of the AsA-GSH pathway. It catalyzes the conversion of H_2O_2 to H_2O and O_2 using AsA as specific electron donor (Asada, 1999). APX, thus, prevents the accumulation of toxic levels of H_2O_2 in the cell. APX belongs to class I peroxidase family of proteins which are characterized by the presence of heme prosthetic groups. APXs are extremely sensitive to the concentration of AsA, which is reflected by the rapid decline in their activity at very low concentration (less than 20 μM) of AsA (Shigeoka et al., 2002). The enzyme has been identified in a number of higher plants, algae and cyanobacteria (reviewed in Caverzan et al., 2012). APX gene family comprises of different isoenzymes with different characteristics. Till date, five APX isoforms namely, cytosolic, mitochondrial, peroxisomal/glyoxysomal and chloroplastic have been identified in plants (Dąbrowska et al., 2007). In *Arabidopsis thaliana*, the reported eight isoenzymes of APX can be categorized into three groups: soluble cytosolic (APX1, APX2, and APX6), microsome–membrane bound (APX3, APX4, and APX5) and chloroplastic (sAPX and tAPX) (Dąbrowska et al., 2007; Panchuk et al., 2002) (**Table 2**). Similarly, the identification of APX gene family in *Lycopersicum esculentum* revealed the presence of seven APX genes: three cytosolic, two peroxisomal, and two chloroplastic (Najami et al., 2008). In *O. sativa*, eight members of the APX gene family have been reported; encoding two cytosolic, two peroxisomal, three chloroplastic, and one mitochondrial isoforms (Texeira et al., 2004, 2006). Mitochondrial APX isoforms have also been reported in *Solanum tuberosum* and *Pisum sativum* (Jimenez et al., 1997; Leonardis et al., 2000).

TABLE 1 | Concentrations of AsA and GSH in different subcellular compartments of cell.

Organelles	[Ascorbate] (mM)	[Glutathione] (mM)
Mitochondrion	10.4	14.9
Chloroplast	10.8	1.2
Nucleus	16.3	6.4
Peroxisome	22.8	4.4
Cytosol	21.7	4.5
Vacuole	2.3	0.08

The intracellular AsA and GSH levels in young rosette leaves of Arabidopsis thaliana plants determined using AsA and GSH specific immunogold labeling (Gest et al., 2013; Zechmann, 2014).

The APX isoforms are stress sensitive and are regulated by nearly all kinds of abiotic and biotic stress conditions (Shigeoka et al., 2002). The expression of APX isoforms can be activated by specific factors such as pathogen attack, mechanical pressure, injury, ultraviolet (UVB) radiation, water deficiency, salt stress, low or high temperature, atmospheric pollution, and excess metal ions (reviewed in Shigeoka et al., 2002; Dąbrowska et al., 2007). The stress conditions also modulate the kinetic properties of the enzyme. For example, the exposure of *A. thaliana* wild type and flavonoid mutant (*tt5*) plants to UVB radiation led to a significant decrease in K_m^{AsA} as well as synthesis of new isoforms of cytosolic APX (Rao et al., 1996). The over-expression of APX has been shown to confer tolerance to various abiotic stresses (Xu et al., 2008; Sun et al., 2010; Sato et al., 2011). For example, *Jatropha curcas* plants over-expressing a chloroplastic APX were found to be salt tolerant (Liu et al., 2014). Similarly, over-expression of the peroxisomal APX from the halophyte *Salicornia brachiata* conferred salt and drought stress tolerance to transgenic *Arachis hypogea* plants (Singh et al., 2014). Transgenic *L. esculentum* plants over-expressing cytosolic APX exhibited improved tolerance to chilling, salinity, heat and UVB stress (Wang et al., 2005, 2006). *A. thaliana vtc* mutants deficient in AsA are reported to be hypersensitive to drought stress (Pastori et al., 2003; Faize et al., 2011).

Monodehydroascorbate Reductase

MDHAR (EC 1.6.5.4) recycles MDHA molecules into AsA. The exposure of plants to environmental stress conditions like high light leads to very quick oxidation of AsA to MDHA in chloroplast (Polle, 2001). It is, therefore, necessary for the survival of plants that MDHA is reduced back to regenerate AsA. In chloroplast, MDHA is reduced to AsA by photoreduced ferredoxin at a high rate and this is likely to constitute the main pathway of AsA regeneration in the vicinity of the thylakoid membrane. Away from the thylakoid membrane, reduction of MDHA can occur via two enzymes in the AsA-GSH pathway; DHAR and MDHAR (Asada, 1999). MDHAR reduces MDHA directly by using NAD(P)H as an electron donor. Alternatively, two molecules of MDHA can react non-enzymatically to generate AsA and DHA. The majority of MDHA is, however, found to be reduced by MDHAR (Polle, 2001). MDHAR enzyme activity is found across the entire plant and animal kingdom. Plant MDHARs exhibit high level of sequence similarity with

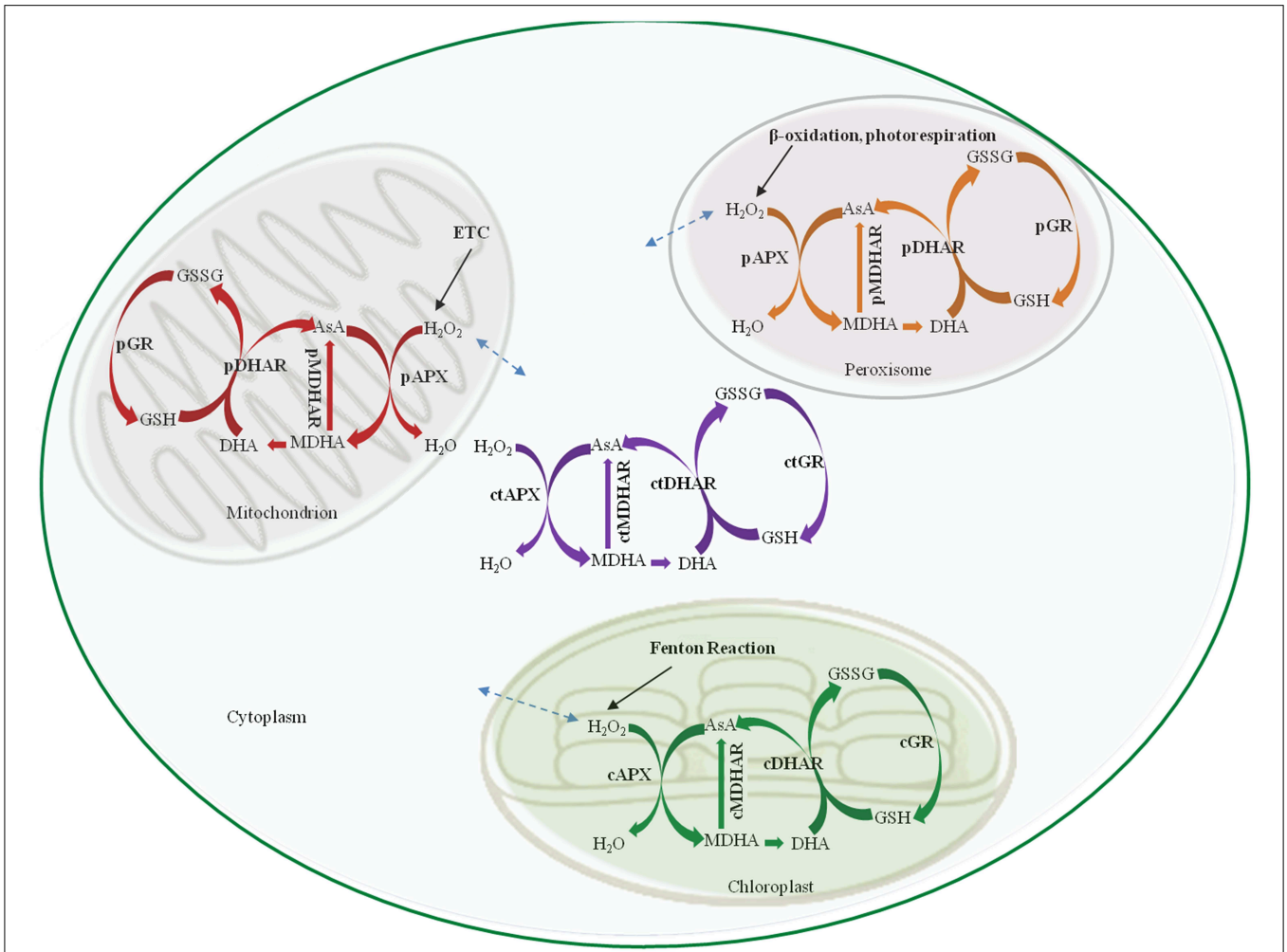

FIGURE 2 | Schematic representation of the AsA-GSH pathway in different sub-cellular compartments. cAPX, cMDHAR, cDHAR, and cGR represent the chloroplastic isoforms; ctAPX, ctMDHAR, ctDHAR, and ctGR stand for the cytoplasmic, mAPX, mDHAR, and mGR indicate the mitochondrial isoforms and pAPX, pMDHAR, pDHAR, and pGR represent the peroxisomal isoforms. H_2O_2 can freely diffuse between the different organelle as indicated by broken arrows. ETC, Electron transport chain; AsA, Ascorbic acid; GSH, Glutathione; GSSG, oxidized glutathione dimer; MDHA, Monodehydroascorbate; DHA, Dehydroascorbate.

prokaryotic flavoenzymes. MDHAR activities are reported to be present in algae (Haghjou et al., 2009), bryophytes (Lunde et al., 2006) and in all higher plants (Yoon et al., 2004; Leterrier et al., 2005). Higher plants' MDHARs belong to a multigene family constituting several sub-cellular isoforms. MDHAR activity has been detected in several cell compartments, such as chloroplasts, mitochondria, peroxisomes and cytosol (Jimenez et al., 1997; López-Huertas et al., 1999; Mittova et al., 2003; Kavitha et al., 2010). In *A. thaliana*, six isoforms of MDHAR are present among which two are peroxisomal, two are cytosolic and one is dually targeted chloroplastic/mitochondrial isoform (Lisenbee et al., 2005) (**Table 2**). The *L. esculentum* MDHAR family consists of three isoforms (Stevens et al., 2007). A total of three cytosolic isoforms of MDHARs have been reported in the moss *Physcomitrella patens* (Lunde et al., 2006). *Physcomitrella* apparently lacks any chloroplastic isoform indicating that AsA reduction in the plant exclusively occurs in cytosol (Drew et al., 2007).

In order to protect against the deleterious effects of ROS, the AsA pools are required to be maintained in a reduced state. Thus, ascorbate reductases like MDHARs, which are responsible for the reduction of AsA have considerable roles in stress tolerance. The activity of MDHAR proteins as well as MDHAR gene expression has been found to be differentially affected by various stress conditions. The increase in MDHAR activity has been reported in stress conditions like salinity, high light, UV radiation, boron toxicity and low temperature (Mittova et al., 2003; Cervilla et al., 2007). Transgenic studies have also confirmed the vital role of this enzyme in conferring tolerance to various abiotic stresses. For example, over-expression of *A. thaliana* MDHAR in *Nicotiana tabacum* enhanced tolerance of transgenic plants to ozone, salt and dehydration stress (Eltayeb et al.,

TABLE 2 | AsA-GSH pathway gene families in *A. thaliana* and *O.sativa* and their role in abiotic and biotic stress tolerance.

Gene	Localization	*O. sativa*	*A. thaliana*	Role in abiotic and biotic stress	References
APX	Cytoplasm	LOC_Os03g17690 LOC_Os07g49400	At1g07890 At 3g09640	Over expression confers tolerance to cold, salt stress and bacterial wild fire disease. Knockdown causes hyper-responsiveness to pathogen attack	Mittler et al., 1999; Wang et al., 2005; Faize et al., 2012
	Peroxisome	LOC_Os04g14680 LOC_Os08g43560 LOC_Os09g36750	At4g35000	Over expression confers salt and drought tolerance	Singh et al., 2014
	Chloroplast Stroma	LOC_Os12g07830 LOC_Os12g07820 LOC_Os04g035520	At4g08390	Over expression confers salt tolerance	Liu et al., 2014
	Thylakoid	LOC_Os02g34810	At1g77490		
MDHAR	Peroxisome	LOC_Os02g47790 LOC_Os09g39380 LOC_Os02g47800	At3g27820 At3g52880	Knockdown leads to enhanced resistance to *P. stritiformis*	Feng et al., 2014
	Chloroplast stroma/Mitochondria	LOC_Os08g05570	At1g63940	Over expression confers heat, salt and oxidative stress tolerance	Li et al., 2010; Eltelib et al., 2012
	Cytoplasm	LOC_Os08g44340	At5g03630 At3g09940	Over expression confers salt tolerance	Sultana et al., 2012
DHAR	Chloroplast	LOC_Os06g12630	At5g16710	Over expression confers salt and cold stress tolerance	Li et al., 2012
	Cytoplasm	LOC_Os05g02530	At1g75270	Over expression confers aluminum stress tolerance	Yin et al., 2010
GR	Cytoplasm	LOC_Os02g56850	At3g24170	Over expression confers oxidative stress tolerance	Aono et al., 1991
	Chloroplast/Mitochondria	LOC_Os03g06740 LOC_Os10g28000	At3g54660	Over expression confers oxidative and cold stress tolerance	Aono et al., 1991; Foyer et al., 1995

The table enlists the representative members of the gene families encoding AsA-GSH pathway with their corresponding locus names, localization details and role in abiotic and biotic stress tolerance.

2007). The over-expression of *Acanthus ebracteatus* cytoplasmic and *Malpighia glabra* chloroplastic MDHAR genes improved salt stress tolerance in *O. sativa* and *N. tabacum*, respectively (Eltelib et al., 2012; Sultana et al., 2012). Similarly, over-expression of chloroplastic MDHAR from *L. esculentum* and *Avicennia marina*, respectively, was shown to confer resistance to high temperature and methyl viologen-mediated oxidative stress in transgenic *L. esculentum* (Li et al., 2010) and to salt stress in transgenic *N. tabacum* plants (Kavitha et al., 2010).

Dehydroascorbate Reductase

AsA, which is a major anti-oxidant in plants, is oxidized to DHA via successive reversible electron transfers with MDHA as a free radical intermediate. DHA, so produced, is reduced to AsA by DHAR with GSH as an electron donor (EC 1.8.5.1). DHAR is the key enzyme to regenerate AsA. DHARs have been isolated and characterized from higher plants like *A. thaliana*, *N. tabacum* and agricultural crops such as *oleracea*, *O. sativa* and *Pennisetum glaucum* (Urano et al., 2000; Shimaoka et al., 2000; Ushimaru et al., 2006; Pandey et al., 2014). In *A. thaliana* five different DHARs (At1g19550, At1g19570, At1g75270, At5g36270, At5g16710) have been identified, with their presence either in an organelle (chloroplast or mitochondrion) or in the cytosol (Chew et al., 2003) (**Table 2**). Recently the At1g19570 isoform has been found to be associated with peroxisomes (Kataya and Reumann, 2010). Two different DHAR isoforms have been discovered in *Spinacia oleracea* leaves with one isoform located in chloroplasts whereas the other being present in the sub-cellular compartment other than chloroplasts (Shimaoka et al., 2000). DHAR activity has also been found in mitochondria, chloroplasts and peroxisomes of both leaf and root cells of the cultivated *L. esculentum* (M82) and its wild salt-tolerant relative, *L. pennellii* (Lpa) (Mittova et al., 2000). Two DHAR genes encoding for cytosolic and chloroplastic DHARs have also been identified in *Eucalyptus* spp. (Teixeira et al., 2005).

DHAR also plays an important role in abiotic stress tolerance and its expression is activated by a number of abiotic stress factors (Ali et al., 2005; Lu et al., 2008; Fan et al., 2014). Moreover, enhanced tolerance to various abiotic stresses was observed in

plants over-expressing DHAR (Kwon et al., 2003; Ushimaru et al., 2006; Wang et al., 2010). For example, the over-expression of *A. thaliana* cytosolic DHAR has been shown to impart tolerance to aluminum stress in transgenic *N. tabacum* plants (Yin et al., 2010). In yet another report, it was shown that the over expression of DHAR which led to enhanced AsA accumulation conferred oxidative and salt stress tolerance to *L. esculentum* plants (Li et al., 2012). The simultaneous expression of chloroplastic *O. sativa* DHAR and *E. coli* GR in *N. tabacum* plants resulted in enhanced tolerance to salt and cold stress (Le Martret et al., 2011).

Additionally, DHAR plays an important role in plant growth and development (Chen and Gallie, 2006). The lack of DHAR resulted in the quick loss of AsA from *O. sativa* plants and led to slower rate of leaf expansion consequently affecting plant growth and development (Ye et al., 2000).

Glutathione Reductase

GR (NADPH: oxidized glutathione oxidoreductase; EC 1.6.4.2) maintains the cellular redox state by regenerating the reduced form of GSH, thereby, maintaining the balance between reduced GSH and AsA pools (Noctor and Foyer, 1998; Reddy and Raghavendra, 2006). GR is a flavo-protein oxidoreductase ubiquitously present in both prokaryotes and eukaryotes (Romeo-Puertas et al., 2006). The protein has been purified and characterized from a variety of organisms (Rao and Reddy, 2008; Achary et al., 2014). Although localized mainly in the chloroplasts, the enzyme is also found in cytosol (Edwards et al., 1990), mitochondria and peroxisomes (Jimenez et al., 1997; Romeo-Puertas et al., 2006).

Multiple isoforms of GR have been reported in a number of plants (Edwards et al., 1990; Lascano et al., 2001; Contour-Ansel et al., 2006; Rao and Reddy, 2008; Trivedi et al., 2013). Modulation in the expression profile of various GR isoforms have been known to occur under various stress conditions (reviewed in Yousuf et al., 2012; Gill et al., 2013). Transgenic *N. tabacum* plants over-expressing *E. coli* GR in the cytoplasm and chloroplast exhibited enhanced GR activity and tolerance to methyl viologen-mediated oxidative stress (Aono et al., 1991, 1993). Similarly, the over-expression of GR in chloroplasts of *N. tabacum* plants led to enhanced accumulation of GSH and AsA and the transgenic plants were found to be more tolerant to high light and chilling stress (Foyer et al., 1995). Overproduction of chloroplastic GR led to reduced photoinhibition under chilling stress in transgenic *Gossypium hirsutum* plants (Kornyeyev et al., 2003). Transgenic *N. tabacum* plants with reduced expression of GR were shown to display enhanced sensitivity to oxidative stress (Ding et al., 2009).

AsA-GSH Pathway in Chloroplasts

The AsA-GSH cycle plays a critical role in maintaining ROS homeostasis in chloroplasts. These organelles are devoid of catalases and the AsA-GSH cycle acts as the major H_2O_2 metabolizing pathway in these photosynthetic organelles. The photoreduction of O_2 in chloroplast via photosystem–I (PSI) leads to the formation of superoxide ions, which rapidly dismutate to H_2O_2 spontaneously or by the action of superoxide dismutases (Asada, 1999). Chloroplasts contain relatively higher levels of AsA and GSH as compared to the other sub cellular organelles (Noctor and Foyer, 1998; Gest et al., 2013; Zechmann, 2014). Thus, the AsA-GSH pathway in chloroplast is imperative in protecting it from the deleterious effects of excess ROS production. Among the four enzymes of the AsA-GSH pathway in chloroplasts, the chloroplastic APX (chAPX) which consists of thylakoid (tAPX) and stromal (sAPX) isoforms scavenges the H_2O_2 generated during photosynthesis. The stromal and thylakoid-bound APXs have been identified and purified from several plant species (Ishikawa et al., 1996, 1998). tAPX is characterized by the presence of an extended C-terminal sequence that makes it 5 KDa larger than the sAPX (Asada, 1999). This sequence is responsible for binding of the protein to the membrane. sAPX has been shown to be predominantly important for photo-protection in young leaves. tAPX and sAPX isoforms are apparently functionally redundant and contribute to oxidative stress tolerance in chloroplasts. A sudden exposure to high light stress in *tapx and sapx* double mutant of *A. thaliana* led to a characteristic decline in the photochemical efficiency of PSII (Kangasjärvi et al., 2008). Likewise, the over-expression of tAPX in *N. tabacum* plants helped in maintaining photosynthetic efficiency of plants under high light and low temperature stress, thereby, substantiating the role of chloroplastic APX in stress resistance (Yabuta et al., 2002). The MDHA formed in the lumen by the oxidation of AsA disproportionates to DHA and moves into the stroma through the thylakoid membrane. MDHA produced by both stromal and thylakoid bound APX isoforms is reduced by stromal MDHAR. MDHAR has not been reported in the lumen of chloroplast (Obara et al., 2002). Along with the regeneration of AsA from MDHA, chloroplastic MDHAR also brings about the photo-reduction of dioxygen to $O_2^{\bullet-}$ in absence of MDHA (Miyake et al., 1998; López-Huertas et al., 1999). DHAR and GR activities convert the DHA translocated from the lumen and the DHA generated in the stroma to AsA (Asada, 1999).

AsA-GSH Pathway in Mitochondria

The presence and activity of AsA-GSH cycle enzymes in mitochondria of plant cells have been established, and this cycle plays an important role in protecting mitochondrion against the toxic ROS regularly produced in respiratory chain reactions (Leonardis et al., 2000; Chew et al., 2003; Mittova et al., 2004; Lázaro et al., 2013). The mitochondrial AsA-GSH cycle deals with both photosynthetic as well as stress-induced oxidative stress (Jimenez et al., 1997). The mitochondrial AsA-GSH cycle also plays an important role in eliminating the mitochondrial-derived radicals, thereby protecting the heme of leghemoglobin in N_2-fixing legume root nodules (Iturbe-Ormaetxe et al., 2001; Loscos et al., 2008). The mitochondrial APX is known to be membrane-localized in plants (Leonardis et al., 2000; Iturbe-Ormaetxe et al., 2001). The best collective evidence for the presence of MDHAR, DHAR, and GR in mitochondria is from *P. sativum* leaves (Jimenez et al., 1997) and *Phaseolus valgaris* nodules (Iturbe-Ormaetxe et al., 2001).

AsA-GSH Pathway in Cytoplasm

In *A. thaliana*, the cytosolic AsA-GSH pathway is characterized by the presence of one cytosolic APX (APX1), with an additional stress inducible APX (APX2) (Panchuk et al., 2002), along with the other enzymes (Mittler et al., 2004). It has been shown that the cytosolic APX imparts cross compartment protection of the other sub-cellular organellar APXs like mitochondrial APX, thylakoidal and stromal APXs hinting toward the fact that cytosolic AsA-GSH pathway plays an important role in protecting the other organelles during periods of stress (Davletova et al., 2005). Notably, cytosolic APX accounts for up to 0.9% of the total soluble protein of nodules and is particularly abundant in infected cells and nodule parenchyma of *Medicago sativa* (Dalton et al., 1998).

AsA-GSH Pathway in Peroxisome

Peroxisomes are single membrane-bound subcellular organelles being involved in production as well as the degradation of H_2O_2 and are sites for photorespiration, fatty acid β-oxidation, glyoxylate cycle and ureide metabolism (Corpas et al., 2001; Mano and Nishimura, 2005). The four enzymes of the AsA-GSH cycle, APX, MDHAR, DHAR and GR have been reported to be expressed in peroxisomes of roots and leaves of *P. sativum* and *L. esculentum* (Jimenez et al., 1997; Mittova et al., 2000; Leterrier et al., 2005). The presence of reduced AsA and GSH, and their oxidized forms, DHA and GSSG, respectively, was demonstrated by high performance liquid chromatography (HPLC) analysis in intact peroxisomes of *P. sativum* leaves (Jimenez et al., 1997). cDNAs encoding peroxisomal APX have been isolated from *Gossypium* spp. (Bunkelmann and Trelease, 1996), *A. thaliana* (Zhang et al., 1997) and *S. oleracea* (Ishikawa et al., 1998). The deduced amino acid sequence of peroxisomal APX has a high degree of identity with cytosolic APX, but it has a C-terminal amino acid extension containing a single, putative membrane−spanning region (Mullen et al., 1999). DHAR and GR were also found in the soluble fraction of peroxisomes, whereas membrane bound APX proteins have been shown to be present in *P. sativum*, *Cucurbita maxima*, and *L. esculentum* (Yamaguchi et al., 1995; Bunkelmann and Trelease, 1996; López-Huertas et al., 1999).

Role of Gene Families of AsA-GSH Pathway in Abiotic Stresses

Drought Stress

Drought stress leads to the production of ROS (mainly H_2O_2) in chloroplasts and mitochondria of plant cells (Dat et al., 2000). Drought stress causes varied effects on the enzymes of the AsA-GSH cycle, the response being dependent on the plant species, the developmental and metabolic state of plant, and the duration and intensity of the stress (Sofo et al., 2010). In majority of cases, drought stress led to an increase in the activity of enzymes of AsA-GSH cycle (Reddy et al., 2004; Sofo et al., 2005; Pukacka and Ratajczak, 2006; Bian and Jiang, 2009). For example, desiccation of recalcitrant seeds of *Acer saccharinum* was characterized by increased O_2^- and H_2O_2 production, elevation in AsA and GSH contents as well as increased activity of the AsA-GSH enzymes (Pukacka and Ratajczak, 2006). Similarly, subjecting five *Morus alba* cultivars to drought stress led to an increase in the activity of AsA-GSH cycle enzymes (Reddy et al., 2004). During prolonged drought treatment in *Prunus spp*, the activities of the AsA-GSH enzymes were up-regulated, AsA/DHA ratio was decreased and the ratio of GSH/GSSG was increased suggesting an important role of the AsA-GSH pathway in combating drought stress (Sofo et al., 2005). Polyethylene glycol (PEG) induced drought stress to *Cucumis sativus* seedling roots led to increased activity of APX. However, the activities of DHAR and MDHAR first decreased (24 h) and then increased. The activity of GR was found to decrease at all time points (Fan et al., 2014). Drought stress differentially affected the antioxidant levels in the genotypes of plants which were contrasting with respect to drought tolerance. For example, the drought tolerant cultivars exhibited enhanced antioxidant enzyme activity under drought stress in comparison with sensitive cultivars of *Dendranthema grandiflorum* (Sun et al., 2013). The effect of drought stress on different isoforms of AsA-GSH cycle genes is extremely variable among different plant species. For example, drought stress was shown to decrease the activity of cytosolic isoform of APX whereas it led to increased activity of the chloroplastic isoform in *Helianthus annuus*. In the same study, it was shown that drought stress did not affect the activity of both the cytosolic and chloroplastic isoforms of APX in *Sorghum bicolor* (Zhang and Kirkham, 1996).

Salt Stress

In plants, salinity stress leads to cellular dehydration, which enhances the production of ROS causing oxidative stress and thereby leading to enhanced expression of ROS scavenging enzymes. The expression levels of all enzymes of AsA-GSH pathway have been shown to be affected by salt stress (Mittova et al., 2004; Jebara et al., 2005). However, activities of AsA-GSH pathway enzymes were found to be differentially altered by salinity stress in the salt tolerant and sensitive varieties. For example, *O. sativa* L. cv. Pokkali which is a salt-tolerant genotype, showed enhanced activity of AsA-GSH cycle enzymes, whereas, the salt-sensitive, *O. sativa* L. cv. BRRI dhan 29 exhibited decreased APX activity, increased DHAR activity and unchanged MDHAR and GR activity (Hossain et al., 2013). However, salinity stress in *Triticum aestivum* and *O. sativa* resulted in increased activities of MDHAR (Sairam et al., 2002; Vaidyanathan et al., 2003). All the isoforms of MDHAR, *viz*. mitochondrial, peroxisomal, chloroplastic, and cytosolic have been found to be sensitive to salt stress. For instance, salinity stress leads to increased activities of mitochondrial and peroxisomal MDHARs in *Lycopersicon pennellii,* which is a salt tolerant wild variety (Mittova et al., 2003). An increased GR activity has been reported in the roots and leaf of *Cicer arientinum* under salt stress (Eyidogan and Oz, 2005).

Temperature Stress

High temperature in plants enhances the generation of ROS, consequently inducing oxidative stress (Yin et al., 2008). Under high temperature, RuBisCO can lead to the enhanced production of H_2O_2 as a result of its oxygenase reaction (Kim and Portis,

2004). Tolerance to heat stress has been ascribed to elevated antioxidant enzymes' activity in many crop plants (Rainwater et al., 1996; Sairam et al., 2000; Sato et al., 2001; Rizhsky et al., 2002; Vacca et al., 2004; Almeselmani et al., 2006). The AsA-GSH pathway was found to be upregulated in response to heat stress in *Malus domestica* as reflected by increased gene expression and activities of APX, DHAR and GR enzymes (Ma et al., 2008). Under heat stress, the response of antioxidant enzymes activity varied amongst different genotypes of plants. For example, the analysis of gene expression of APX in a thermo-tolerant and thermo-susceptible variety of *Brassica* spp, *T. aestivum*, and *Vigna radiata* revealed increased activity of the enzyme under heat stress in all the genotypes. However, the elevation in transcript level was found to be higher in case of thermo-tolerant genotypes (Almeselmani et al., 2006; Rani et al., 2013). Heat stress induced elevation in transcript level of APX has also been reported in *Poa pratensis* by He and Huang (2007). Similar to APX, GR activity was also found to be enhanced by 50% in thermo-tolerant and 33% in thermo-susceptible genotypes of *Brassica* spp under heat stress (Rani et al., 2013). Exposure of *N. tabacum* cell suspension to elevated temperature (55°C) also resulted in increased GR activity (Locatto et al., 2009). However, Ma et al. (2008) reported the initial increase and then decrease in GR activity in *M. domestica* leaves during prolonged exposure to heat stress. The activities of DHAR and GR were also found to be increased under heat stress in temperature sensitive orchid *Phalaenopsis* (Ali et al., 2005). The activity of MDHAR was found to be repressed under heat stress in the same study. This study also indicated a differential effect of heat on the activity of the antioxidant enzymes in roots and shoots. For example, the activity of GR was doubled at 40°C in leaf but was drastically reduced in roots at the same temperature. The authors attributed the decrease in GR activity in roots to reduced availability of NADPH (Ali et al., 2005).

Similar to heat stress, low temperature stress also induces H_2O_2 production in cells (Suzuki and Mittler, 2006) and is known to up-regulate transcripts, protein level and activities of different ROS-scavenging enzymes (Prasad et al., 1994; Saruyama and Tanida, 1995; Sato et al., 2001). In *Pinus* spp, enhanced freezing tolerance during cold acclimation was characterized by elevated levels of APX, GR, MDHAR, and DHAR (Tao et al., 1998). In leaves of *Eupatorium adenophorum*, the activity of APX and GR increased with decreasing temperatures. However, upon cold stress treatment to leaves of thermo-tolerant *E. odoratum*, the activity of APX reached a peak value at 15°C and then declined, whereas GR activity was not affected. MDHAR activity in leaves of the cold-treated *E. adenophorum* was not significantly different from the controls, whereas the activity was found to be decreased in leaves of *E. odoratum*. DHAR activity in leaves of the two species was found to increase with both heat and cold stresses (Lu et al., 2008).

Role of Gene Families of AsA-GSH Pathway in Biotic Stress

The production of ROS constitutes one of the first responses of plant cells to infection (Torres et al., 2006). The apoplastic generation of ROS occurs mainly by enzymes like membrane NADPH-dependent oxidase, cell wall peroxidase or polyamine oxidases (Bolwell et al., 2002). ROS generated upon pathogen attack can either enhance the harmful effect of infection or may contribute to plant defense by causing hypersensitive response (Levine et al., 1994). ROS can also serve as signal molecules for the activation of local and systemic resistance (Grant and Loake, 2000; Kuzniak and Skłodowska, 2005). The ROS-mediated plant defense response is further more complex and is dependent on factors like the life style of pathogen (biotrophy/necrotrophy), the type of plant–pathogen interaction (compatible/incompatible interactions) and the stage of plant development (Govrin and Levine, 2000; Huckelhoven and Kogel, 2003). For maintaining ROS homeostasis, it becomes important to have an intricate and tightly regulated balance between ROS production and removal. Pathogen induced changes in antioxidant enzyme levels have been shown in a number of plants (**Table 3**). For example, in *Hordeum vulgare* leaves challenged with the powdery mildew fungus, *Blumeria graminis*, the fungal infection led to a significant decrease in APX and GR activity in whole-leaf extracts

TABLE 3 | Representative examples of modulation of plant antioxidant activities by different pathogens.

Pathogen	Plant	Effect of pathogen infection on the enzymes of AsA-GSH cycle	References
Blumeria graminis	*Hordeum vulgare*	APX, GR activity decreased while MDHAR and DHAR unaffected	Vanacker et al., 1998
Botrytis cinerea	*Lycopersicon esculentum*	Activity of chloroplastic APX, GR increased and that of mitochondrial and peroxisomal isoforms decreased	Kuzniak and Skłodowska, 2005
Alternaria sesami	*Sesamum orientale*	Activity of APX, MDHAR, and GR initial increased and then decreased	Shereefa and Kumaraswamy, 2014
Piriformospora indica	*Arabidopsis thaliana*	Activity of cytosolic MDHAR and DHAR increased	Vadassery et al., 2009
Mycosphaerella fragariae	*Fragaria ananassa*	Activity of APX, MDHAR, and GR increased.	Ding et al., 2011
Fusarium oxysporum	*Cicer arientinum*	Increased activities of APX and GR	Limones et al., 2002
Erwinia amylovora	*Malus domestica*	Decreased APX activity in chloroplasts	Viljevac et al., 2009

of resistant variety but caused no significant change in the susceptible one. However, there was no change in the activities of MDHAR and DHAR (Vanacker et al., 1998). Kuzniak and Skłodowska (2005) showed that *Botrytis cinerea* infection differentially affected the AsA-GSH gene families in *L. esculentum*. Upon infection, APX activity was found to increase in chloroplasts and decrease in mitochondria and peroxisomes 2 days after infection (dpi). The activity of peroxisomal MDHAR increased considerably at 1 dpi followed by subsequent decrease in activities of all MDHAR isoforms. A significant reduction in the activity of DHAR was observed in whole leaf extract at all time points. The chloroplastic DHAR activity was not affected, whereas the mitochondrial and peroxisomal DHAR activities were distinctly decreased starting from the third day after pathogen challenge. The GR activity on the other hand was found to increase in the chloroplasts. The peroxisomal and mitochondrial GR activities were repressed in response to infection by the pathogen. The decline in the activity of mitochondrial and peroxisomal isoforms points toward the "fungus-promoted precocious senescence" that led to the disease development (Kuzniak and Skłodowska, 2005). Similarly, *Sesamum orientale* plants, upon infection with the fungus *Alternaria sesami* displayed an initial increase in the activity of APX, MDHAR, and GR followed by a gradual decrease in the corresponding activities (Shereefa and Kumaraswamy, 2014). The expression of cytosolic MDHAR and DHAR was shown to be upregulated in *A. thaliana* seedlings co-cultivated with the root-colonizing endophytic fungus *Piriformospora indica* suggesting an important role of the enzyme in the maintenance of mutualistic plant- fungal interaction (Vadassery et al., 2009). However, knockdown of *T. aestivum* MDHAR resulted in improved resistance to *Puccinia striiformis* in wheat (Feng et al., 2014) suggesting that plants with compromised activity of the antioxidant enzymes have improved resistance against pathogens.

Role of Gene Families of AsA-GSH Pathway in Physiological and Developmental Processes of Plants

Apart from the important role in protecting the plants from the stress induced ROS, the enzymes of AsA-GSH pathway also play a part in growth and development of plants. AsA and GSH have been known to play important roles in organ developmental processes of plants (Arrigoni and De Tullio, 2002). The peroxisomal MDHAR in *A. thaliana* has been shown to be important in mobilization of lipid reserves during early growth following germination by removing H_2O_2 generated by β-oxidation (Eastmond, 2007). The transcript profiles of certain enzymes of the pathway are known to be spatially and developmentally regulated. Expression of *A. thaliana* cytosolic APX (APX1) in leaves and roots is relatively high as compared to the cytosolic APX2 isoforms (Panchuk et al., 2005; Hruz et al., 2008). *A. thaliana apx1* mutant plants exhibit delayed development, late flowering and altered stomatal responses (Pnueli et al., 2003). The study of Correa-Aragunde et al. (2013) suggests the participation of APX1 in the redistribution of H_2O_2 accumulation during root growth and lateral root development in *A. thaliana*. The transcripts of APX1 in *Ipomoea batata* were detected clearly in leaves, weakly in stems, and not in non-storage and storage roots. The expression level appeared to be higher in mature leaves than in immature leaves, suggesting its growth-stage specific expression (Park et al., 2004). Expression of APX2, another cytosolic isoform was found to be limited to bundle sheath cells in leaves exposed to excess light (Fryer et al., 2003). Like APX, DHAR also plays an important role in developmental processes. It has been reported that suppression of DHAR expression results in a preferential loss of chlorophyll a and less CO_2 assimilation, resulting in decreased rate of leaf expansion, reduced foliar dry weight and premature leaf aging. Furthermore, the over-expression of DHAR which led to reduced lipid peroxidation in the transgenic plants led to delayed leaf aging in *O. sativa* (Chen and Gallie, 2006).

Summary and Perspectives

Despite their deleterious effects, ROS at low concentrations play crucial roles in stress perception, regulation of photosynthesis, pathogen recognition, programmed cell death, and plant development. The antioxidant enzymes of AsA-GSH pathway help in maintaining ROS homeostasis in cells by avoiding the potential cytotoxicity of ROS and allowing them to function as signal molecules. Considering the different levels and intensities of AsA and GSH production in the different organelles of cell under normal and stress conditions, the regulation of antioxidant enzymes also differs. There are different subcellular isoforms of each of the antioxidant enzymes and each isoform differentially responds to different stress and developmental cues. The mechanism of regulation of each isoforms by different stresses and developmental stages is yet to be completely understood. Further studies are required to decipher the complex regulation of expression of different isoforms of the AsA-GSH pathway enzymes in order to bolster our understanding of ROS homeostasis in plants. Understanding the intricate regulation of the various isoforms under various stress conditions can facilitate deeper insights into the stress tolerance mechanism of plants. This will also help in designing better strategies for the development of plants with improved abiotic and biotic stress tolerance.

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