The Role of Ascorbic Acid in Plant–Pathogen Interactions

Hatem Boubakri

Abstract Ascorbic acid (AsA) is one of the most abundant antioxidant molecules in plants. AsA provides the first line of defense against damaging reactive oxygen species (ROS), protecting plant cells from many environmental factors that induce oxidative stress, including wounding, ozone, high salinity, and pathogen attacks. AsA interacts with key elements of a complex network orchestrating plant defense mechanisms, thereby influencing the outcome of plant–pathogen interaction. It can act in coordination with glutathione (GSH) and important enzymatic antioxidants in the AsA-GSH cycle to provide the appropriate redox environment regulating diverse defense pathways such as the expression of defense genes through the activation of the NPR1 (Nonexpressor of Pathogenesis-Related protein 1) regulatory transcription factor, the strengthening of cell walls, and the modulation of defense-hormonal signalling networks. On the other hand, AsA was found to act either as an inducer *per se* or as a component of induced resistance (IR) process to pathogens when elicited by other inducers/elicitors such as β-aminobutyric acid (BABA, a non-proteinic amino acid), jasmonic acid (JA) and its methyl ester (methyljasmonate, MEJA), and extracellular polysaccharides (EPSs). This chapter provides a broad picture on the mechanisms by which AsA interacts with key components of a complex network regulating both basal and induced resistance in different pathosystems.

Keywords Ascorbic acid · Biotic stress · Hormonal-signalling · Redox-related defense responses · AsA-GSH cycle · Basal resistance · Induced resistance

1 Introduction

Plants are exposed to attack by an immense array of pathogen microorganisms (viruses, fungal, bacteria, and insect herbivores). To counteract these invaders and protect themselves, they developed sophisticated strategies and complex molecular

H. Boubakri (✉)
Laboratory of Leguminous, Centre of Biotechnology of Borj-Cédria, Hammam-Lif, Tunisia
e-mail: hatem.boubakri@cbbc.rnrt.tn

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mechanisms (Zipfel 2009; Jones and Dangl 2006). Some of these defense-related traits are expressed by a plant constitutively, regardless of a plant's history of attack by pathogens such as the preformed physical barriers in the leaf surface formed by the cuticle (Zipfel 2009). Besides of these pre-existing defense barriers, plants can activate structural and chemical defense mechanisms after pathogen attacks (Jones and Dangl 2006). In incompatible interactions, conserved microbial structures (pathogen-associated molecular patterns, PAMPs) were recognized by plants via transmembrane receptors (pattern recognition receptors, PRRs), triggering intracellular-defense mechanisms (Jones and Dangl 2006). This process, known as PAMP-triggered immunity (PTI), is manifested by extracellular alkalinization, protein phosphorylation, defense gene upregulation, and the generation of reactive oxygen species (ROS). These immune-defense responses are responsible for limiting pathogen spread (Ryals et al. 1996). In the case of compatible interaction, the control of the adapted pathogens is achieved by multiple applications of chemicals known for their negative effect on human health (Boubakri et al. 2016). Thus, the use of disease resistance inducers to protect plants from pathogens has gained important attention by the scientific community on the aim to develop a new alternative strategy to these chemicals in controlling plant diseases. This strategy relies on the manipulation of the natural host-defense repertoire known as "induced resistance" (IR) (Ryals et al. 1996; Walters et al. 2013). IR is characterized by an increased manifestation of plant innate-defense responses against different pathogens triggered by the application of various external factors (Garcia-Brugger et al. 2006; Pieterse et al. 2013). IR implies a complex signalling network involving salicylic acid (SA) defense-signalling pathways (Pieterse et al. 2013) and jasmonic acid (JA)-dependent and ethylene (ET)-dependent signalling pathways (Van Wees et al. 2008), and requires a functional *NPR1* (Nonexpressor of pathogenesis-related protein 1) regulatory gene for the induction of defense genes (Ryals et al. 1996). After pathogen attacks, plants respond by expressing a wide battery of defense-related genes and that in both basal and induced resistance processes. One of these responses is the oxidative burst, a rapid production of ROS. Although they have antimicrobial activity *per se* and can therefore reduce pathogen growth, they also contributed to the damage of crop plants (Lamb and Dixon 1997). However, hydrogen peroxide (H_2O_2) has been shown to play a central role in the expression of disease resistance in different pathosystems. It serves as a substrate for the oxidative cross-linking of cell wall components, and it plays a key role in defense signalling leading to the induction of PR genes mainly through activation of NPR1 transcription factor (Lamb and Dixon 1997). Plants have evolved efficient antioxidant systems to cope with toxicity of ROS (Khan et al. 2012). This includes lipid-soluble membrane-associated antioxidants, such as α-tocopherol and β-carotene, and water-soluble antioxidants, like glutathione (GSH) and ascorbate (AsA) (Khan et al. 2012). A major H_2O_2 detoxifying system in plant cells is the ascorbate-glutathione (AsA-GSH) cycle involving successive oxidations and reductions of AsA and GSH catalyzed by the enzymes constituting the cycle, namely ascorbate peroxidases (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Pignocchi et al. 2003). In the recycling pathways,

which work to maintain the AsA pool size, the oxidized forms of AsA, monodehydroascorbic acid (MDHA) and dehydroascorbic acid (DHA), are reduced back to AsA by MDHAR and DHAR, respectively (Gallie 2013).

Besides its direct antioxidant role, AsA is involved in ROS sensing and defense response signalling. Several studies described that the levels of AsA increased following pathogen attacks (Tsuda et al. 2005; Fujiwara et al. 2013). AsA accumulation promoted oxalic acid (OA) biosynthesis leading to an accumulation of H_2O_2, which is an important defense signalling molecule in plants (Dias et al. 2011). Such increase of H_2O_2 levels influenced the redox state of the cell environment which induced changes in the conformation of the NPR1 transcription factor leading to PR gene expression (Mou et al. 2003; Pavet et al. 2005). Several lines of evidence suggest that the interaction between ROS, AsA and GSH, mediated by the AsA-GSH cycle, and the link between the antioxidative metabolism and redox-related defense responses have a key role in defense signalling mechanisms during both basal and induced resistance in plants. Even though that changes in AsA contents during plant growth and the role of AsA in abiotic stress conditions have been well documented, its function under biotic constraints has not been fully characterized.

2 Key Insights from Studies of Mutations in AsA Synthesis

To gain insights on the role of AsA in plant defense mechanisms, several mutants with altered levels of AsA have been obtained. Seven ascorbate-deficient *vtc* mutants have been identified and represent four different *VTC* loci (Conklin et al. 2000). *VTC1* encodes GDP-mannose pyrophosphorylase (Conklin et al. 1999) and *VTC4* encodes L-galactose 1-P phosphatase (Conklin et al. 2006), which are both enzymes in the proposed GDP-mannose pathway for AsA biosynthesis (Wheeler et al. 1998). *VTC2* encodes GDP-L-galactose phosphorylase, also a biosynthetic enzyme (Dowdle et al. 2007). The identity and function of *VTC3* are unknown. The AsA contents in the *vtc* mutant lines range from 50% of wild-type levels in *vtc2-3*, *vtc3*, and *vtc4* to 25–30% in *vtc1-1*, *vtc1-2*, *vtc2-1*, and *vtc2-2*. The study of some physiological parameters indicated that *vtc1-1* and *vtc2-1* mutant lines have a slower growth rate (Conklin et al. 2000; Pastori et al. 2003; Pavet et al. 2005), but without any effect on photosynthesis rate (Müller-Moulé et al. 2003) in plants grown under moderate light intensity. Additionally, some changes in the intracellular distribution of antioxidant enzymes associated to AsA have been described, but the overall capacity of the antioxidant system is largely unchanged, except for a marked increase in nonspecific peroxidase activity (Conklin et al. 1999). Unperturbed overall leaf antioxidant capacity is indicated further by leaf H_2O_2 contents in the mutant, which are similar to those in the wild type (Veljovic-Jovanovic et al. 2001). Despite this fact, *vtc1* is smaller than the wild-type plant and shows retarded flowering and accelerated senescence (Veljovic-Jovanovic et al. 2001). Because *vtc1* does not accumulate H_2O_2 and the redox states of leaf antioxidants are not changed (Veljovic-Jovanovic et al. 2001), these phenotypic effects are linked to modified amounts of

vitamin C rather than to a general change in cellular redox balance. Thus, this mutant provides an excellent system for studying the effects of physiologically relevant decreases in AsA level.

Transcriptomic analysis on the *vtc1-1 A. thaliana* mutant line compared with the wild type using microarray technology allowed the identification of 171 genes that were differentially expressed in *vtc1-1*; of these, 12.9% were genes involved in defense mechanisms. In particular, PR proteins showed a relatively high level of induction (Pastori et al. 2003). Abscisic acid (ABA) concentration was 60% higher in vtc1-1 than in the wild type, so ABA signalling may provide a link between ascorbate levels and PR protein transcript levels. Other experiments also suggested that AsA-deficient mutants have increased SA, an increased transcript level of genes encoding PR proteins, peroxidase activity, and accumulation of the phytoalexin camalexin (Barth et al. 2004; Pavet et al. 2005). In addition, *vtc1-1* and *vtc2-1* mutant lines both showed increased resistance to infection by virulent pathogens (Barth et al. 2004; Pavet et al. 2005). In fact, an enhanced resistance to *Pseudomonas syringae pv. maculicola* ES4326 and to the oomycete *Hyaloperonospora parasitica pv.* Noco was noted in both *vtc1-1* and *vtc2-1* mutant lines (Pavet et al. 2005; Barth et al. 2004). However, contrarily to the results obtained by Pastori et al. (2003), Barth et al. (2004) reported that PR proteins were not more highly expressed in *vtc1-1* compared to the wild type, but were induced more strongly by pathogen infection, due to higher levels of SA.

Pavet et al. (2005) concluded that AsA deficiency induced PR gene expression through an enhanced GSH accumulation and higher redox levels (more GSH than GSSG). The authors, however, suggested that H_2O_2-signalling and SA-signalling pathways are not implicated in this process (Fig. 1). More recently, Mukherjee et al. (2010) described that the enhanced resistance to *P. syringae* in the AsA-deficient *A. thaliana vtc1-1* mutant correlates with elevated levels of SA, which induced the expression of *PR* genes via the NPR1 pathway. The authors suggested that AsA deficiency causes constitutive priming via a buildup of H_2O_2 that stimulates SA accumulation, leading to an increased disease resistance (Fig. 1). This supports previous findings describing that AsA deficiency led to the expression of PR genes in a SA-dependent manner and the premature senescence positively contributed to this offered resistance (Kus et al. 2002). On the other hand, Botanga et al. (2012) described that the *Arabidopsis vtc1* and *vtc2* mutants were more sensitive to the pathogenic ascomycete *A. brassicicola*. It seems that the deficiency in AsA could either increase or decrease disease resistance levels depending on the pathogen lifestyle.

3 ROS Neutralization by AsA in Response to Pathogen Attacks

One component of plant defense is the generation of ROS, which are mainly produced by NADPH oxidase, amine oxidase, and peroxidases located mainly extracellularly (Kuzniak 2010). The generation of ROS is associated with the induction of

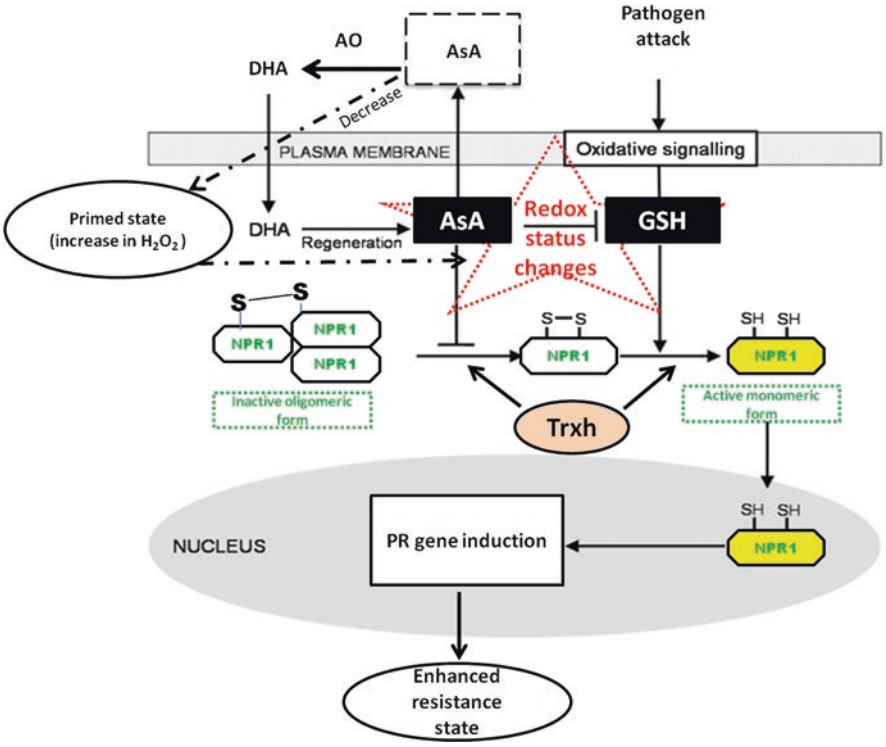

Fig. 1 Key insights from *vtc* mutant *A. thaliana* lines on the role of AsA on defense signalling in response to pathogen attacks. Arabidopsis mutant lines (*vtc*) showed a deficiency in AsA content and increased levels of GSH compared to the wild type (Pastori et al. 2003; Pavet et al. 2005). The apoplastic ascorbate redox state depends on the balance between ascorbate oxidation to DHA by AO and cytosolic regeneration by reduction of DHA by GSH (Pavet et al. 2005; Foyer and Noctor 2005). It was suggested that this increase in GSH content in mutant lines provided an appropriate redox state in the cell environment for activating the NPR1 regulatory transcription (Pastori et al. 2003; Mou et al. 2003). This enables the reduction of cysteine residues in NPR1 by a thioredoxin h-type (Trxh). Thus, NPR1 is reduced from an inactive oligomeric form localized in the cytosol to an active monomeric form through the reduction of intermolecular disulfide bonds (Tada et al. 2008). Monomeric NPR1 is then translocated into the nucleus activating the expression of PR genes, thereby providing an enhanced resistance state to pathogen attacks (Pavet et al. 2005; Tada et al. 2008). It was also suggested that AsA deficiency in *vtc* mutant lines created a priming state characterized by increased levels of H_2O_2 (dashed arrows) which means changes in the redox state of the GSH pool which can easily shift to its oxidized form, GSSG, leading to the activation of NPR1 transcription factor through the SA-signalling pathway (Mukherjee et al. 2010). *AO* ascorbate oxidase, *DHA* dehydroascorbate, *MDHA* monodehydroascorbate, *GSH* glutathione, *GSSG* glutathione disulfide, *AsA* ascorbate, *NPR1* nonexpressor of pathogenesis-related protein 1, *Trxh* thioredoxin h-type, *SA* salicylic acid, H_2O_2 hydrogen peroxide

programmed cell death (PCD), which has been shown to restrict the growth of biotrophic fungal pathogens (Kuzniak 2010). Rapid infection-induced production of ROS in the apoplast could also inhibit pathogen penetration by cell wall lignification, cross-linking of cell wall polymers (Almagro et al. 2009) as well as by the production of antimicrobial phytoalexins (Lamb and Dixon 1997). In addition, ROS have the potential to be directly toxic to pathogens and to induce the expression of defense genes (Desikan et al. 2001). Furthermore, compartmentalization of both ROS production and activation of antioxidants was found to contribute to fine-tuning of ROS levels and their signalling properties (Torres et al. 2006). Although the primary oxidative burst following pathogen recognition occurred in the apoplast, ROS are produced in other organelles with oxidative metabolism components or characterized by rapid electron flux, e.g., chloroplasts, mitochondria, and peroxisomes may also have functions in defense (Kuzniak 2010). Thus, ROS are main players contributing to the inducible defense responses to pathogen attacks; however, their amounts in plant cells are strictly regulated to prevent plant tissue damage by a sophisticated antioxidant system (Desikan et al. 2001; Torres et al. 2006).

In plants, AsA plays an important role on the detoxification of ROS either directly or through the AsA-GSH cycle. The AsA-GSH cycle was found to be functional in different cellular compartments including the apoplast, cytosol, chloroplasts, mitochondria, and peroxisomes (Noctor and Foyer 1998). AsA could be found either in a reduced form (AsA) or in two oxidized forms (MDHA and DHA). A remarkable accumulation of ROS was observed in plant cells after pathogen attack, as well as large amounts of DHA as a result of AsA oxidation. For example, in tomato (*Solanum lycopersicum*) fruits, AsA levels rose ~40% after infection with an attenuated strain of *Cucumber mosaic virus* (Tsuda et al. 2005). In addition, an increase in AsA levels was observed in *Brassica rapa* resistant cultivars after infection with *Turnip mosaic virus (*TuMV) (Fujiwara et al. 2013). However, a susceptible Chinese cabbage cultivar showed a significant decrease in AsA content in the inoculated leaves. These results suggest that AsA accumulation is not induced in the compatible interactions between *B. rapa* and TuMV (Fujiwara et al. 2013). The mechanisms underlying AsA accumulation in response to pathogen infection are still unknown. Although, it has been demonstrated that the AsA level was positively correlated with the extent of viral resistance, negative correlations has also been reported. For example, an AsA deficiency can increase resistance to other pathogens such as *P. syringae* and *P. parasitica* in *vtc* mutant lines of *Arabidopsis* (Barth et al. 2004).

It was suggested that the increase in AsA amounts after pathogen infection shifts the AsA pool towards a more oxidative state (Foyer and Noctor 2005). In this reaction, the reduced form of AsA was oxidized to MDHA. Thereafter, MDHA was either reduced by MDHAR to AsA or, because it is very unstable, reacted to DHA. DHA is reduced by DHAR to AsA. In this reaction, the reduced form of glutathione (GSH) is oxidized to glutathione disulfide (GSSG). GSSG is then reduced by glutathione reductase (GR) to GSH. The electron acceptor NADP is regenerated during the reduction of MDHA and GSSG by the respective enzymes (Noctor and Foyer 1998; Foyer and Noctor 2005). The regeneration of the AsA pool (reduced form) through the different enzymatic recycling pathways (DHAR and MDHAR) is

needed to restore the antioxidative capacity of plant cells. In other terms, the ratio between reduced and oxidized AsA is pivotal for the ability of the plant to fight oxidative stress (Noctor and Foyer 1998; Foyer and Noctor 2005). Additionally, some reports described that both pathogen attacks and treatments with disease resistance inducers can change total AsA contents in plants which makes AsA an important stress marker during biotic stress situations (Khan et al. 2012; Tsuda et al. 2005; Boubakri et al. 2016).

4 Host-Innate Defense Facets Regulated by AsA-Related Redox Status

Important defense facets which are known to be expressed after pathogen attacks could be influenced by AsA-related redox status, including the induction of PR genes after changes in the conformation of the NPR1 regulatory transcription factor (from the S–S form to the SH–SH form), strengthening of the cell wall, and modulation of defense-hormonal signalling pathways.

4.1 NPR1 Transcription Factor Activation

AsA is a main cellular redox buffer and the related redox signalling contributed to the orchestration of plant defense responses (Dias et al. 2011; Khan et al. 2012; Bala and Thukral 2011). In fact, through its interplay with GSH in the AsA-GSH cycle, AsA can modulate the ROS signalling in different cellular compartments and determine its intensity, duration, and localization, i.e., the ROS signature (Mittler et al. 2004), that can determine in turn the type and intensity of the response to a specific pathogen (Kuzniak 2010).

The increase in AsA content following pathogen attacks favored an accumulation of oxalic acid (OA) and, thereby, an accumulation of H_2O_2 because OA is a major contributor in the biosynthesis of H_2O_2. The increase in H_2O_2 amounts influenced the redox status of the GSH pool that can easily shift to its oxidized form, GSSG (Pavet et al. 2005). Knowing that the redox state of GSH is concentration-dependent, changes in the content of GSH even with constants GSH/GSSG ratios would alter the cellular redox state, thereby, activating redox-related defense reactions (Fig. 1). To date, one of the best known examples of how the GSH-related redox signalling pathways may work, inducing alterations in plant genes expression is the activation of NPR1 protein (Mou et al. 2003).

NPR1 is present in the cytosol as an oligomer with subunits aggregated via disulfide bonds (Tada et al. 2008). The TGA1 transcription factor bound to the promoter of SA-responsive gene (TGACG) is not competent to activate defense genes, exemplified as PR1. Mobilization of enzymatic and nonenzymatic antioxidant

mechanisms results in a shift of the cellular redox environment towards reducing conditions. This enables the reduction of cysteine residues in NPR1 and TGA1, by a thioredoxin h-type (Trxh). Thioredoxins are small proteins catalyzing thiol-disulfide interchange in the target proteins. Thus, NPR1 is reduced from an inactive oligomeric complex localized in the cytosol to an active monomeric state through the reduction of intermolecular disulfide bonds (Tada et al. 2008). The reduction of NPR1 preceding defense gene induction requires an increase in GSH content and a concomitant shift in the cellular redox environment towards the reducing conditions (Mou et al. 2003; Fobert and Despres 2005). Monomeric NPR1 is then translocated into the nucleus where it interacts with transcription factors of the TGA class, such as TGA1 and TGA2 (Fig. 1) (Mou et al. 2003; Pieterse and Van Loon 2004).

NPR1 is an intrinsic component of the SA-defense signalling pathway, during both basal and induced resistance in plants. The redox dependence of the NPR1 pathway implied that biotic stimuli that perturb the cellular redox state upregulated defense gene expression via the NPR1 pathway (Mou et al. 2003; Pieterse and Van Loon 2004). Interestingly, the kinetics of the changes in the GSH pool and the GSH/GSSG ratio required for *in vitro* NPR1 reduction were similar to those evoked during IR process (Mou et al. 2003). Collectively, it seems that the AsA and the antioxidative enzymes undergo dynamic changes under biotic stress modulating the redox state of GSH, thereby, providing the appropriate cellular redox environment for the activation of NPR1 transcription factor.

4.2 Cell Wall Strengthening

The products of plant metabolism are separated from pathogen invaders by the cuticle and cell wall. When pathogens attempt to breach these barriers for colonization, plants respond by expressing several wall-associated defense reactions. The most known cell wall-related defense responses include the inhibition of fungal cell wall-degrading enzymes, secretion of fungitoxic peptides and phytoalexins, and cell wall strengthening via a lignification process (Huckelhoven 2007). Cell wall lignification is a complex process occurring exclusively in higher plants; its main function is to strengthen the plant vascular body. This process involved the deposition of phenolic polymers, the so-called lignins, on the extracellular polysaccharidic matrix. These polymers arise from the oxidative coupling of three cinnamyl alcohols in a nonrandom reaction, in which cell wall polysaccharides appear to influence the freedom of cinnamyl alcohol radicals, being a highly orchestrated process (Hagemeier et al. 2001). In basal resistance, lignification makes the cell wall more resistant to mechanical pressure applied during penetration by fungal appressoria (Huckelhoven 2007). Additionally, a lignified cell wall is water resistant and thus less accessible to cell wall-degrading enzymes.

The findings by Takahama and Oniki (1992) indicated that the apoplastic AsA pool had an important role in the lignification process after pathogen perception. These authors reported that AsA in its reduced form negatively regulated the

peroxidase-dependent oxidation of phenolics by the reduction of the oxidized phenolic intermediates. They suggested that the lignification process occurred when the apoplastic AsA pool was largely oxidized. It was shown that in a resistant tomato cultivar the level of reduced AsA in the apoplast decreased significantly after infection with *Botrytis cinerea*, and this response was correlated with high peroxidase activity (Kuzniak 2010). The importance of the cell wall strengthening processes mediated by the apoplastic class III peroxidases for plant resistance to infection has been identified in different pathosystems (Lamb and Dixon 1997; Almagro et al. 2009). Similarly, the ascorbate-deficient (*vtc*) *A. thaliana* mutants with increased resistance to infection by virulent pathogens (Barth et al. 2004; Pavet et al. 2005) exhibited elevated cell wall peroxidase activity (Colvile and Smirnoff 2008). Therefore, AsA was considered as a major redox active compound in the apoplast and it was found to play an important role after pathogen attacks when the invader was perceived firstly by the apoplast. In general, the apoplastic AsA pool accounts between 5 and 10% of the total cellular pool (Noctor et al. 2002; Noctor and Foyer 1998; Veljovic-Jovanovic et al. 2001).

The redox status of the apoplastic AsA pool is regulated by ascorbate oxidase (AO) (Pignocchi et al. 2003; Sanmartin et al. 2003). This enzyme is considered the first step in the AsA degradation pathway in the apoplast (Sanmartin et al. 2003). The presence of AO may also explain why AsA in the apoplast is markedly more oxidized than cytoplasmic AsA (Pignocchi et al. 2003). AO has long been considered to influence cell expansion via the modulation of redox state of the apoplast (Smirnoff and Wheeler 2000), although the exact mechanism is still largely unknown. It has been suggested that the controlled oxidation of apoplastic AsA via AO could have a similar effect on the apoplastic redox state as an oxidative burst (Foyer and Noctor 2005).

On the other hand, several lines of evidence indicated that the concentrations of AsA and GSH and their redox ratios might also have an important role in both apoplastic metabolism and plant defense. Indeed, the apoplastic levels and redox status of AsA and GSH were described to be influenced during both compatible and incompatible interactions of barley with *Botrytis graminis*. Moreover, the activities of several extracellular antioxidative enzymes were increased upon powdery mildew attack (Noctor et al. 2002; Vanacker et al. 1998, 2000). In general, the activities of the antioxidative enzymes in the apoplast are low compared to those recorded in the cytosolic compartment; however, they can significantly affect the cell wall's redox status, thereby influencing the cell wall rigidity, because AsA, H_2O_2, and hydroxyl radicals had an important role in cell expansion (Smirnoff and Wheeler 2000). Clearly, the balance between AsA and H_2O_2 is a determinant factor for the extent of lignification of the cell wall. Indeed, *Arabidopsis* plants over-expressing AO accumulated the oxidized form of AsA in their apoplast and were found to be less responsive to auxin and more susceptible to the virulent *P. syringae* (Huckelhoven 2007). In addition, both an increase in mitogen-activated protein kinase (MAPK) activity and altered gene expression were observed in the lines with oxidized apoplastic AsA. This suggests that the apoplastic redox status could also modulate pathogen responses following a translation into cytoplasmic signalling. In fact,

different reports indicated that direct oxidation of apoplastic AsA can change the outcome of a plant–pathogen interaction via a cytoplasmic response to the apoplastic redox status (Kuzniak 2010). The homeostasis of AsA and GSH in the apoplast is easily perturbed as this compartment lacks a regeneration system to restore the reduced forms. Regarding the low amount of the total AsA and GSH in the apoplast and its low buffering capacity, the specific plasma membrane transport systems, linking the apoplastic AsA and GSH redox couples to their cytoplasmic counterparts, appear to play a key role in maintaining the AsA-GSH cycle-dependent defensive responses of this compartment during the plant–pathogen interaction (Foyer and Noctor 2005; Kuzniak 2010). The regulation of the apoplastic redox status seems to be important at the point of orchestrating key cytoplasmic defense mechanisms by providing optimum levels of ROS. Eventually, ROS are involved in defense gene expression, phytoalexin production, and cell death (Boubakri et al. 2012, 2013a, b).

4.3 Modulation of Defense-Hormonal Signalling Pathways

Plant responses to biotic and abiotic stresses are mediated by various hormones. Hormones function in a complex signalling-network modulating plant defense response to various external factors. Biotic stress signalling in plants involved mainly four hormones which are: SA, JA, Et, and abscisic acid (ABA). SA regulated defense-signalling to many biotrophs, whereas JA and Et are involved in resistance to necrotrophs, although there are many exceptions (Glazebrook 2005). The induction of SA promotes dissociation of NPR1 oligomers into monomers in the cytosol through reduction of disulfide bonds between the NPR1 monomers, which then enter the nucleus (Despres et al. 2003; Mou et al. 2003). Phytohormones were found to act in an antagonistic or synergistic manner, allowing some of them to prevail on others (Suza et al. 2010). In fact, SA, JA, and Et exhibited antagonistic interactions. The activation of the SA-signalling pathway repressed the JA/Et pathway through NPR1 and WRKY70 transcription factors, and the ABA pathway through NPR1 or its downstream elements. The induction of the JA/Et pathway inhibited the expression of certain defense-related genes regulated by the SA-signalling pathway via MAPK4 (Mitogen-Activated Protein Kinase 4) (Glazebrook 2005; Suza et al. 2010).

A unifying feature of the diverse stresses that activate JA signalling is that they all generate ROS accumulation, and JA signaling-deficient mutants exhibited an enhanced susceptibility to oxidative stress (Dombrecht et al. 2007; Suza et al. 2010). In addition, jasmonates are known to induce ROS production when applied exogenously (Zhang and Xing 2008) as well as when produced *de novo* in response to leaf wounding (Suza et al. 2010). Thus, JA likely plays a role in regulating the redox balance of stressed plant tissues. Given that JA and AsA both contribute to plant defenses against oxidative stresses, the potential interaction between these two factors is very possible and merits further investigation. In addition, AsA was reported to play an important role in the biosynthesis of certain plant hormones, including Et

and gibberellic acid (GA), and cell wall glycoproteins and secondary metabolites with antimicrobial properties (Suza et al. 2010). Further, the quantity and redox state of AsA may also influence JA signalling. For example, in tomato, silencing of the terminal enzyme in the Man/Gal pathway resulted in a decrease in the ratio of AsA to DHA and also increased several JA-responsive transcripts such as proteinase inhibitors and arginine decarboxylase (Suza et al. 2010). Pathogen attacks induced changes in the levels of AsA in plants (Sasaki-Sekimoto et al. 2005; Wolucka et al. 2005; Tsuda et al. 2005). For example, Tsuda et al. (2005) reported an accumulation of AsA levels after viral infection in tomato (*Solanum lycopersicum*) fruits. In fact, AsA content rose ~40% after infection with an attenuated strain of *Cucumber mosaic virus* (genus *Cucumovirus*, family *Bromoviridae*, order unassigned). Abscisic acid (ABA) concentration was 60% higher in *vtc1-1* than in the wild type, so ABA signalling may provide a link between AsA levels and PR protein transcript level. Other experiments have shown that AsA-deficient mutants have increased SA content, an increased transcript level of genes encoding PR proteins, peroxidase activity, and accumulation of the phytoalexin camalexin (Pavet et al. 2005).

5 Involvement of AsA in the Establishment of Induced Resistance (IR)

5.1 AsA as a Component of IR Process

Several reports have described the effect of different inducers/elicitors of disease resistance on AsA metabolism in plants. Elicitors are known to induce host-defense responses by mimicking a pathogen attack or other stress, and can be substances of pathogenic origin or compounds released by the plants in response to the action of a pathogen (Boubakri et al. 2013a). Elicitors such as JA and its methyl ester (methyljasmonate, MEJA), SA, BABA (β-aminobutyric acid), and EPSs (extracellular polysaccharides) have been shown to influence AsA metabolism when exogenously applied.

Both JA and MEJA are known to be potent elicitors of innate-defense responses in several plant species (Turner et al. 2002; Almagro et al. 2014). Jasmonates (JAs) application induced a transcriptional reprogramming leading to an enhanced resistance to various pathogens (Almagro et al. 2014; Turner et al. 2002). It was reported that treatment of *N. tabacum* and *A. thaliana* suspension cells with MEJA stimulated the *de novo* biosynthesis of AsA (Wolucka et al. 2005). On the basis of transcript profiling data, it was found that this AsA accumulation in tobacco cells was accompanied with an important induction of *VTC1* gene encoding a GDP-mannose pyrophosphorylase, a key enzyme in AsA biosynthesis pathway (Wolucka et al. 2005). However, it is not yet clear whether induction of AsA by exogenous JAs is a direct JA signalling response that requires all components of JA synthesis and signalling, or is triggered by an effect of JA treatment such as ROS generation, which is caused by but not specific to JA.

SA is known to regulate plant innate-defense responses to several pathogens, mainly the biotrophic fungi, through NPR1 pathway and to induce defense responses and resistance to various pathogens when exogenously applied through eliciting the SAR-related molecular mechanisms (Sudhamony et al. 2009). It was reported that SA treatment induced AsA accumulation in *A. thaliana*; however, the molecular mechanisms underlying such event are still unknown (Suza et al. 2010).

In addition, BABA, a non-proteinic amino acid known to be the most efficient priming agent of SAR in plants, induced *VTC1* expression in *Arabidopsis*, upon infection with *Plectosphaerella cucumerina*, while *APX1* transcription was inhibited (Pastor et al. 2013). This might lead to a more oxidized environment in the cell and allow increased ROS accumulation. Additionally, in BABA-treated plants, the level of mRNA transcripts of *VTC1* gene was enhanced, but not that of *GSH1* gene (Pastor et al.2013). This transcriptomic regulation would lead to a more oxidized state in the cytoplasm by the augmented H_2O_2 accumulation as suggested by the lower levels of GSH/GSSG in BABA-treated plants following *P. cucumerina* inoculation (Pastor et al. 2013). As mentioned above, the cytosolic thiol-disulfide status plays an important role in regulating PR gene expression through NPR1 transcription factor which might affect the outcome of the plant–pathogen interaction (Mou et al. 2003).

The EPSs (extracellular polysaccharides) that modulate the activity of PAL (phenylalanine ammonia-lyase), which is the first enzyme in the phenylpropanoid pathway, also induced AsA accumulation (Mittler 2002). In addition, these phytopathogenic molecules decreased the levels of cytosolic APX which is known to detoxify ROS using AsA as substrate. However, the amount of DHA increased after treatments of cells with the active EPSs. This suggests that an increasing amount of ASC has been used by different metabolic pathways (Mittler 2002). As the activity of APX was decreased by EPSs, the level of ROS increased and thereby an increase in DHA amount. The changes in the redox balance of the ASC/DHA pair and in the activities of the AsA-redox enzymes specifically induced by these phytopathogenic molecules suggest an involvement of these molecules in plant–pathogen interactions. Further, cell death is known as an important event during both incompatible plant–microbe interactions and IR process. It is initiated by the interaction between the *R* gene product and the *Avr* gene product and leads to the restriction of pathogen spreading (Boubakri et al. 2016). The establishment of cell death in EPS-treated cells was preceded by changes in the cytosolic APX activity and a decrease in the total AsA pool (AsA + DHA), as well as a remarkable shift of this redox pairs towards the oxidized form (de Pinto et al. 2002). Overall, these findings suggest an important role for AsA during the establishment of IR by elicitors from different origins.

5.2 AsA as an Inducer of Disease Resistance

Besides its role as a component of IR process, AsA was found to act as an inducer of disease resistance in some pathosystems (Egan et al. 2007; Fujiwara et al. 2013). *Turnip mosaic virus* (TuMV) is known to infect a wide range of hosts including

economically important *Brassica* species (Walsh and Jenner 2002). Recently, it was reported that the exogenous application of DHA and AS derivatives l-(+)-ascorbic acid 2-sulfate disodium salt dehydrate (AS-SO_4) and fat-soluble ascorbyl palmitate (AS-Pal) induced resistance to *TuMV* in turnip (*B. rapa* subsp. *rapa*) plants (Fujiwara et al. 2013). Additionally, AsA was shown to be active not only against viruses but also against other pathogens. For instance, treatment of the rice blast fungus *Magnaporthe oryzae* with AsA decreased the percentage of normal appressorium formation (Egan et al. 2007). Further, the simultaneous application of JA and AsA enhanced the accumulation of sakuranetin, a phytoalexin identified from blast resistance rice cultivars, compared to JA alone (Tamogami et al. 1997). Li et al. (2016) reported that the exogenous application of AsA at 600 μM induced resistance in citrus plants against Huanglongbing (HLB), the most devastating disease worldwide under greenhouse and field conditions. Besides, AsA-IR to HLB was accompanied with enhanced traits of the citrus fruit (yield and quality) under field conditions (Li et al. 2016). On the other hand, AsA might possess direct antifungal activities; for example, Botanga et al. (2012) described that AsA application directly affected the hyphal development of *Alternaria brassicicola*. Overall, these findings indicated that AsA had both direct and indirect effects in plant responses to pathogens.

6 Conclusion

Besides its role in ROS neutralization following pathogen attacks, AsA was found to regulate other important host-defense reactions. In basal resistance, based on transcriptomic and genetic approaches, AsA was found to act in coordination with GSH in the AsA-GSH cycle and with the respective antioxidative enzymes providing the appropriate redox environment for the activation of plant defense responses. The modulation of AsA amounts in different cell compartments after pathogen infection influences the redox state of the cell environment, leading to: the expression of various defense responses such as the induction of PR genes through the NPR1 pathway, cell wall strengthening, and the modulation of defense-hormonal signalling pathways. In addition, AsA was shown to be an important component of IR process when elicited by different inducers. On the other hand, AsA was found to act as an inducer of disease resistance mechanisms in various pathosystems. Nevertheless, and contrarily to its role in abiotic stress which is largely detailed and understood, knowledge on the role of AsA in biotic stress is rudimentary. Thus, further studies on how the AsA works in the AsA-GSH cycle to influence and interact with other important components of the innate defense system based on AsA-related redox changes are of great interest.

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