Ascorbate-Glutathione Cycle and Biotic Stress Tolerance in Plants

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Abstract The ascorbate-glutathione cycle (AsA-GSH cycle) is a central pathway of the plant cells linking the H_2O_2-scavenging activity to redox signalling. Here, we summarize the most recent advances in our understanding of the role of AsA-GSH cycle in plant–pathogen interactions. Special attention is paid to the regulatory functions of the AsA-GSH cycle components in plant defence against pathogens, their cross talk with other stress signalling pathways and the functional differences between the cellular compartments in relation to the ascorbate and glutathione-dependent protective systems. As under field conditions, different stresses are likely to occur simultaneously, the involvement of AsA-GSH cycle in the signalling network that regulates the response of plants to a combination of pathogen infection and abiotic stress is also addressed.

Keywords Ascorbate-glutathione cycle · Plant–pathogen interactions · Redox signalling · Compartmentation of stress responses · Stress combinations

1 Introduction

Plants are continuously exposed to a broad range of stress factors which substantially limit crop productivity worldwide. In the nearest future, environmental stresses are predicted to become more severe and widespread owing to the climate change. Under natural conditions, combinations of two or more stress factors,

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acting simultaneously or sequentially, are common to many agricultural areas. The global climate change is leading to the emergence of new and complex stress combinations, and there is growing concern about their impact on crop production. One of the major threats of global warming is the establishment of "new" pests and pathogens extending their geographic range as climatic conditions become more favourable to them (Luck et al. 2011; Atkinson and Urwin 2012; Suzuki et al. 2014). Thus, a detailed understanding of how plants respond to environmental stress factors is not only of fundamental, but also of practical importance since it is necessary to sustain crop productivity and to further support programmes aimed at improving stress tolerance of crop plants.

Environmental stresses alter the production of reactive oxygen species (ROS) in plant cells and influence the interplay between ROS generation and scavenging mechanisms (Muneé-Bosch et al. 2013). A key role in the antioxidant system is attributed to the ascorbate-glutathione cycle (AsA-GSH cycle) which components operate in all compartments of the plant cell. The AsA-GSH cycle is considered to control ROS level through its scavenging activity as well as to contribute to redox sensing and signalling (Foyer and Noctor 2003). The AsA-GSH cycle activity strongly influences the steady-state level of ROS in cells as well as the duration, localization, and amplitude of ROS signals, referred to as ROS signature, which determines the specificity of ROS signalling (Foyer and Noctor 2009; Kuźniak 2010; Shigeoka and Maruta 2014). As ascorbate and glutathione, the main redox buffers of the plant cell, interact with numerous compounds, the ROS-induced changes in their pools can be sensed and transduced to other redox-sensitive signalling pathways, e.g. those mediated by phytohormones such as salicylic acid (SA) and abscisic acid (ABA). Thus, these antioxidants constitute a part of a much complex signalling network that regulates plant growth and stress responses. Traditionally, the imbalance between ROS generation and detoxification was regarded as a major cause of the oxidative damage under stressful conditions and plant resistance was positively linked to the capacity of the antioxidant system. In a wider context, however, the AsA-GSH cycle plays a key role in the plant redox signalling network which controls almost all aspects of plant biology, including defence responses to biotic stress (Foyer and Noctor 2011; Noctor et al. 2017).

2 The Ascorbate and Glutathione-Related Redox Modules

The redox environment of a cell is defined by the redox potential of each redox couple, the pH, and the concentrations of the oxidized and reduced forms of the species comprising redox couples (Schafer and Buettner 2001). The overall redox environment of cells is determined by the equilibrium between ROS and the antioxidant system. The AsA-GSH cycle occurring throughout the plant cell, recycles AsA and GSH and detoxifies H_2O_2. The reduced forms of ascorbate and glutathione are regenerated by the cycle of enzymatic reactions which uses NADPH as the reducing power. Thus, the key redox couples of plant cells, ascorbic acid/

dehydroascorbic acid (AsA/DHA), reduced glutathione/glutathione disulphide (GSH/GSSG) and NADPH/NADP are linked through the AsA-GSH cycle (Noctor 2006). It acts as the main ROS-scavenging and signalling pathway, and ascorbate and glutathione, remain one of the major redox managers in the plant cell and key factors in tolerance against stress. Ascorbate and glutathione can either react with redox active compounds or donate electrons or reducing equivalents to enzymatic reactions (Foyer and Noctor 2011). The metabolic pools of ascorbate and glutathione are tightly linked by the action of enzymes constituting the AsA-GSH cycle, i.e. ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). In the AsA-GSH cycle, APX utilizes two molecules of ascorbic acid (AsA) to reduce H_2O_2 to water with concomitant production of monodehydroascorbic acid (MDHA). APX is a widely distributed antioxidant enzyme, as five different isoforms have been detected at distinct subcellular localizations, namely in the cytosol, chloroplast stroma and thylakoids, peroxisomes, and mitochondria. It has higher affinity to H_2O_2 than catalase. The Km value for catalase is 20–25 mM H_2O_2, while the Km values of chloroplast APX are in the micromolar range; for stromal and thylakoid isoforms, they are 80 and 23 μM, respectively (Miyake and Asada 1992). This makes APX an efficient scavenger of H_2O_2 involved in the subtle regulation of its level. MDHA, an APX-produced radical with a short lifetime, either disproportionates spontaneously to DHA and AsA or is reduced to AsA by a NADPH-dependent enzyme, MDHAR. The latter is the only known enzyme which uses an organic radical as a substrate. In chloroplasts, the reduction of MDHA can occur enzymatically, by MDHAR or directly by ferredoxin. The ferredoxin-mediated reduction of MDHA competes with the photosynthetic electron transfer to $NADP^+$ and prevents NADPH formation. The rate of MDHA reduction by ferredoxin is 30 times higher than that of $NADP^+$ (Miyake and Asada 1992), and it seems that ferredoxin-mediated ascorbate regeneration in chloroplasts has priority over reduction of CO_2 in the Calvin cycle (Backhausen et al. 2000). DHA, a short-lived chemical, is reduced by DHAR using GSH as an electron donor. Otherwise, at pH values greater than 6.0, DHA is easily decomposed to tartare and oxalate. GSH can also reduce DHA non-enzymatically. This reaction occurs at pH values greater than 7 and at GSH concentration higher than 1 mM; hence, it may be important in chloroplasts (Sharma et al. 2012). GSSG produced in this cycle is then converted back to GSH by the action of GR, at the expense of NADPH. As NADPH is consumed in the regeneration process of oxidized ascorbate, reductions in assimilate supply to the pentose phosphate pathway which occur under stress, may affect the antioxidant potential of the AsA-GSH cycle. Moreover, this process competes with other NADPH-dependent reactions that contribute to the plant defence against pathogens such as membrane repair processes and biosynthesis of secondary metabolites, e.g. phenols, lignin, and phytoalexins (Doubnerová and Ryšlavá 2011).

Ascorbate and glutathione are the main hydrophilic antioxidants and redox buffers in plant cells and their buffering capacity maintains the overall cellular homeostasis. Ascorbate is present in millimolar concentrations and the GSH content is usually about tenfold lower (Noctor 2006). In plant cells, the ascorbate and glutathi-

one pools are highly reduced in the cytosol, chloroplasts, and mitochondria. They are oxidized in the apoplast and vacuoles, organelles lacking efficient mechanisms to regenerate AsA and GSH from their oxidized forms (Foyer and Noctor 2011; Shigeoka and Maruta 2014; Noctor and Foyer 2016). The levels and redox ratios of ascorbate and glutathione depend on the balance between the rates of their biosynthesis and turnover mediated by the enzymes of the AsA-GSH cycle. The repeated redox-cycling of these antioxidants allows to regulate the cellular redox state which is particularly important for plant survival strategies under stress (Paciolla et al. 2016; Noctor et al. 2017).

Ascorbate and glutathione are found in all cellular compartments and cell types. More than 90% of ascorbate is located in the cytosol, determining the redox buffering capacity of this compartment. A considerable part of its pool is exported to the apoplast, where it is found in millimolar concentration (Conklin 2001; Arrigoni and De Tullio 2002; Foyer and Noctor 2011). At this location, ascorbate is considered to be the only significant redox buffer (Pignocchi and Foyer 2003). In *Arabidopsis*, the immunocytochemical labelling revealed the highest levels of ascorbate in peroxisomes and the cytosol and the lowest one in vacuoles. The intermediate labelling was found in nuclei, mitochondria, and plastids (Zechmann et al. 2011). Under high light conditions, however, a significant part of the ascorbate pool is located in the vacuoles where it helps to reduce the phenoxyl radicals produced by H_2O_2-mediated oxidation of phenols (Takahama 2004). The content of glutathione in *Arabidopsis* cells was found to be highest in mitochondria followed by nuclei, the cytosol, peroxisomes, plastids, and vacuoles (Zechmann et al. 2008; Zechmann 2014).

The biological role of ascorbate and glutathione in plants has been intensively studied; however, their functions in defence against pathogens seem to be underexplored. The cellular ascorbate and glutathione contents and their redox ratios have been showed to contribute in different ways to pathogen defence. Owing to their antioxidant properties, they can protect cell compartments from oxidative damage. Ascorbic acid scavenges H_2O_2, superoxide anion (O_2^-), and singlet oxygen ($^1O_2$) in the hydrophilic environments of the cell. The broad antioxidant function of AsA is related to its ability to donate electrons in many non-enzymatic and enzymatic reactions. It is a co-substrate for 2-oxoacid-dependent dioxygenases; thus, it is involved in the biosynthesis of ethylene, flavonoids, anthocyanins as well as hydroxyproline-rich proteins which are involved in the cross-linking of the cell wall after pathogen attack (Zhang 2013). Ascorbate regulates the accumulation of mRNA for enzymes involved in anthocyanin and flavonol synthesis (Page et al. 2012). In the chloroplasts, it is a cofactor for violaxanthin de-epoxidase in the xanthophyll cycle which dissipates excess light energy. It also maintains α-tocopherol in the reduced state. Moreover, by keeping the prosthetic metal ions in reduced forms, AsA maintains the activity of antioxidant enzymes (Arrigoni and De Tullio 2002; Zhang 2013). The studies of Monteiro et al. (2007) revealed a previously uncharacterized antioxidant function of AsA. The authors showed that 1-Cys peroxiredoxin from *A. thaliana* uses ascorbate as reductant although peroxiredoxins have been described to be strictly dependent on thiols. They also suggested that the

ascorbate-dependent peroxidase activity of 1-Cys peroxiredoxin can be relevant for cellular redox processes in plants due to high concentration of AsA in all compartments and the role of peroxiredoxins in redox signalling.

The antioxidant role of glutathione is related to the reduction of ROS and indirectly, to its involvement via the AsA-GSH cycle in reduction of DHA to AsA and in maintaining zeaxanthin and α-tocopherol in the reduced states. It is also a substrate for glutathione-S-transferases (GST) and glutathione peroxidases, enzymes which participate in the xenobiotic detoxification, reduction of lipid hydroperoxides, and in ROS removal. The involvement of GSH in the antioxidant defence mechanisms is also related to its interactions in a reversible manner with protein cysteinyl thiols, a process known as glutathionylation. The binding of a glutathione to a thiol group protects the protein from irreversible inactivation or regulates protein activity (Rouhier et al. 2008). It has been shown that GST from *Arabidopsis* undergoing glutathionylation possesses DHAR activity (Dixon et al. 2005).

Several studies have shown that there is a close relationship between increased level of H_2O_2 in plant tissues and the glutathione status (Willekens et al. 1997; Rizhsky et al. 2002; Queval et al. 2009). Thus, the status of glutathione pool is now recognized as a marker of oxidative stress triggered by overaccumulation of H_2O_2 and other peroxides which is a hallmark of biotic stress-induced changes in plants (Wojtaszek 1997; Muckenschnabel et al. 2001; Chojak-Koźniewska et al. 2017). The major pathways of H_2O_2 metabolism mediated by glutathione which could be important for plant defence against pathogens involve three distinct types of peroxidases, namely APX, thiol peroxidases such as peroxiredoxins and GST. APX is functionally linked to glutathione through the AsA-GSH cycle. Peroxidases from the peroxiredoxins family can use GSH alone or via the action of glutaredoxin (GRX) and some GSTs possess a GSH-dependent peroxidase activity (Rouhier et al. 2008). These two pathways of glutathione peroxidation are independent of the AsA-GSH cycle per se; however, their activities depend on the availability of GSH regenerated via the cycle and they are involved in defence against pathogens. Transcripts of certain GSTs appear to be useful indicators of the level of intracellular H_2O_2 generated during oxidative stress and they can be induced by SA, a known mediator of plant response to biotrophic pathogens (Queval et al. 2009; Sappl et al. 2009).

The AsA-GSH cycle is an antioxidant pathway but it also regulates the signalling potential of ascorbate and glutathione. They are sensors of the environmental changes transducing them into redox signals and co-ordinating the response. To date, photosynthesis is the best understood metabolic pathway in plant cells governed by redox control. However, it is becoming increasingly evident that many other cellular processes in plants are subject to redox regulation which is a universal, fine-tuning mechanism involved in adjusting the whole-cell metabolism to the constantly changing environment (Fedoroff 2006).

The most studied redox system in photosynthetic organisms is the thioredoxin system; however, emerging evidence indicates that GRXs which sense the GSH/GSSG ratio could also be important in redox signalling in plants (Rouhier et al. 2008). Thioredoxins interact with numerous proteins involved in a broad range of

physiological processes and regulate their activity. Two enzymes of the AsA-GSH cycle, APX and DHAR, have been identified as targets of thioredoxins (Dos Santos and Rey 2006). The chloroplast thioredoxins are linked to ferredoxin but the cytosolic ones are reduced by NADPH; thus, they are associated with the NADP-glutathione-ascorbate network. The NADP-dependent thioredoxin reductases transfer the reducing power of NADPH to the thioredoxin/peroxiredoxin system for scavenging ROS (Cha et al. 2015). Their functional involvements under stress conditions, however, have not been elucidated. In *Arabidopsis*, the cytosolic thioredoxin *AtTRXh5* gene was found to be up-regulated during incompatible interactions with the bacterial pathogen *Pseudomonas syringae*, indicating its possible implication in response to pathogens (Laloi et al. 2004).

There are two classical, well-characterized examples of the involvement of redox reactions in plant defence against pathogens, namely programmed cell death (PCD) manifested by the hypersensitive response (HR) and the SA-regulated pathogenesis-related (PR)-1 gene expression.

PCD is induced by pathogens at the sites of infection through a mechanism known as HR, and is one of the best-known defence responses mediated by redox signals. HR is assumed the most common defence reaction in resistance against avirulent pathogens with an obligatory biotrophic lifestyle. It results in rapid killing of infected plant cells and the neighbouring, non-infected ones, ultimately leading to death of the invading pathogen which requires living host cells for growth and reproduction. Moreover, HR induces signals that spread into more distant tissues and trigger defence mechanisms (Greenberg 1997; Kombrink and Schmelzer 2001; Greenberg and Yao 2004). Several necrotrophic fungi, however, induce PCD as an invading strategy to kill the plant cells and feed on the dead tissues (Govrin and Levine 2000).

ROS, especially H_2O_2, act as key signals in plant PCD. The generation of ROS during the HR follows a biphasic pattern. The low-amplitude first phase of ROS production is induced by PAMPs (Pathogen-Associated Molecular Patterns) and the second phase with massive accumulation of ROS results from the interaction between the pathogen *avr* gene products and the plant *R* genes (Torres et al. 2006). The kinetics and amplitude of the second rise of ROS, which depends on the balance between ROS production and scavenging, influence the rate of plant cell death and the defence reactions (Mur et al. 2000). Thus, the interplay between ROS and antioxidants determines the redox environment suitable for PCD. A pivotal role in promoting the oxidative conditions needed for HR has been reported for cytosolic APX. Tobacco plants infected with tobacco mosaic virus and *P. syringae* pv *phaseolicola* activated PCD through a signalling pathway involving post-transcriptional down-regulation of cytosolic APX, a key H_2O_2-detoxifying enzyme (Mittler et al. 1998).

Several studies have linked the oxidative cell death mechanism with the contents and redox status of ascorbate and glutathione. The level of GSH has been shown to increase during HR and the glutathione pool in the apoplast, an interface which mediates the first cross talk between host and pathogen, was maintained in the oxidized state (May et al. 1996; Vanacker et al. 1998). Moreover, the HR was

suppressed when bacterial pathogens were applied together with GSH (Mur et al. 2005). Glutathione deficiency of the *Arabidopsis pad2-1* mutant impaired H_2O_2 production in response to the oomycete *Phytophthora brassicae* and correlated with reduced HR (Dubreuil-Maurizi et al. 2011).

The role of glutathione in the regulation of PR1 expression is related to changes in redox state of the NPR1 (Non-expressor of Pathogenesis-Related 1) protein and its translocation from the cytosol to the nucleus to induce SA-dependent transcription of PR proteins (Mou et al. 2003). NPR1 protein is a redox-sensitive, transcriptional cofactor which mediates the transmission of SA signal in plant defence responses. The output of the defence response was found to be determined by the interplay between H_2O_2, SA, and GSH. The SA-regulated transcriptional regulation of defence genes has been found to be a biphasic process. The first, oxidative phase, manifested by increased accumulation of ROS, especially H_2O_2, and decreased GSH-dependent reducing power, is followed by a reductive phase characterized by enhanced GSH content and reducing capacity. These temporal redox changes regulate the conformation of NPR1, a master regulator of SA-mediated defence genes (Mou et al. 2003; Wu et al. 2012). The SA-mediated monomerization of NPR1, conditioning its transfer to the nucleus, is catalysed by thioredoxins (Tada et al. 2008). The NPR1-dependent plant defence signal transduction pathway exemplifies a mechanism by which nuclear NPR1 level is regulated by SA that mirrors the cellular redox state represented by the glutathione redox ratio. The synergism between GSH and SA was shown in transgenic tobacco with increased GSH level which synthesized more SA, was resistant to *P. syringae,* and expressed genes of the NPR1-mediated SA signalling pathway (Ghanta et al. 2011).

PCD is also influenced by ascorbate-dependent redox regulations. The ascorbate-deficient *Arabidopsis* mutants, *vtc1* and *vtc2*, activated localized PCD and had enhanced basal resistance to *P. syringae*. The *vtc1* and *vtc2* mutations enhanced GSH/GSSG ratio and led to constitutive expression of pathogenesis-related (PR) defence genes. The adjustments in the glutathione pool resulting from ascorbate deficiency in the *vt*c mutants, namely the increased GSH/GSSG ratio together with low redox buffering capacity, favour the monomerization and nuclear translocation of NPR1 and facilitate systemic acquired resistance (SAR) responses, for which NPR1 is a master regulator (Pavet et al. 2005).

Interestingly, some natural compounds that induce resistance by a priming mechanism have been shown to control the cellular redox environment after infection (Aranega-Bou et al. 2014). For example, hexanoic acid protected tomato plants against *Botrytis cinerea* by activating a set of detoxifying and redox balance-related genes, including several glutathione transferases, peroxidases, and GR as well as by increasing the ascorbate and glutathione-reduced/-oxidized ratios (Finiti et al. 2014). Thus, hexanoic acid treatment provided a less oxidized environment after infection which might be critical for limiting the growth of necrotrophic pathogens which are known to stimulate ROS generation to their own benefit (Govrin and Levine 2000). In the interactions with necrotrophic pathogens, the reduction of antioxidant capacity followed by increased oxidation and cell death may contribute to susceptibility. Other priming agents, such as thiamine, riboflavin, and chitosan can

also modulate the cellular redox status to protect plants against pathogens (Aranega-Bou et al. 2014).

The ROS signals resulting from the ROS/antioxidants interplay can be transmitted locally, from cell to cell, and spread systemically, throughout the plant in the form of the autopropagating ROS wave. The autopropagating nature of the ROS wave signal means that each cell along its path independently activates its own RBOH (Respiratory Burst Oxidase Homologue) enzyme at the plasma membrane and generates ROS in the apoplast. The ROS wave may therefore spread a stress signal from its initiation site to the systemic tissues (Mittler et al. 2011; Gilroy et al. 2014).

An important aspect of the signalling function of glutathione is related to its involvement in thiol–disulphide interactions in many processes, including gene expression. These interactions can be mediated by GRXs, which reduce disulphide bridges with the help of two glutathione molecules and are key enzymes for the plant response to environmental constraints (Rouhier et al. 2008). It has been shown that GRXs regulate the activity of basic leucine zipper-type transcription factors called TGA which interact with NPR1 and are essential for the regulation of many SA-responsive genes, such as the PR1 gene. In *Arabidopsis*, Ndamukong et al. (2007) identified GRX interacting with TGA factors which was transcriptionally activated when SA content was elevated. Zhou et al. (2000) found that mutations in NPR1 which impaired SA signalling in *Arabidopsis* inhibited the interaction of NPR1 with two transcription factors from the TGA family, TGA2 and TGA3. As these factors were also shown to bind the SA-responsive element of the *Arabidopsis* PR-1 promoter, the results directly link NPR1 to SA-induced PR-1 expression through TGA transcription factors.

3 The Ascorbate-Glutathione Cycle in Plants Under Biotic Stress

The ROS-antioxidant perturbations constitute one of the first responses of plants to infection and antioxidants have been shown to have an underlying influence on plant defence mechanisms against a variety of pathogens. Changes in the cellular contents of ascorbate and glutathione as well as the activities of AsA-GSH cycle enzymes have been reported in numerous studies. Early reports showed that the induced resistance of melon and tomato plants against *Fusarium oxysporum* was accompanied by an increase in the content of GSH (Bolter et al. 1993) and the incompatible tomato–*Cladosporium fulvum* interaction was characterized by a marked accumulation of glutathione, especially in the form of GSSG (May et al. 1996). In the barley-powdery mildew pathosystem, the accumulation of GSH and the activation of some AsA-GSH cycle enzymes were observed during the incompatible interaction and not the compatible one, whereas the content of AsA decreased in both (El-Zahaby et al. 1995). In maize genotypes resistant to *Fusarium,* the activities of the AsA-GSH cycle enzymes were higher than in the susceptible

ones (Lanubile et al. 2012), confirming that the susceptibility to pathogens of different cultivars of the same plant species correlate with the activity of the AsA-GSH cycle.

Changes in the total contents of ascorbate and glutathione as well as in their redox states depending on the type of the plant–pathogen interaction have been also reported in tomato plants infected with *P. syringae* pv *tomato*. A sustained reduction in GSH pool size and redox state concomitant with slight AsA content increase were observed in the susceptible tomato line. In interaction with the resistant cultivar, the glutathione pool homeostasis was maintained and no effect on ascorbate was observed (Kuźniak and Skłodowska 2004b). Similarly, a progressive decrease in the GSH content accompanied by a relative stability in AsA concentration was observed in *B. cinerea*-infected tomato leaves (Kuźniak and Skłodowska 1999). The authors suggested that the shortage of GSH supply could be a limiting factor for operation of the AsA-GSH cycle at the advanced stage of this interaction, when the disease symptoms were the severest, indicating the breakdown of defence mechanisms. In general, the accumulation of GSH is considered to occur in incompatible (plant resistance to pathogen invasion) interactions of plants with pathogens (Gullner and Kömives 2001). As incompatible interactions are usually accompanied by a marked oxidative burst (Wojtaszek 1997), the increased GSH level could reflect an enhanced demand for antioxidant protection or be related to the involvement of GSH in the redox signalling network underlying plant defence responses (Dubreuil-Maurizi and Poinssot 2012; Noctor et al. 2012). In contrast, decreased concentration of AsA, at least transient, appears to be necessary for the elicitation of defence reactions (De Gara et al. 2003; Foyer and Noctor 2005). In a model interaction of *Lotus japonicus* with a non-pathogenic *P. syringae* strain, reduction of ascorbate concentration was suggested to play an important role in plant defence by favouring ROS accumulation. The concentration of glutathione, however, was maintained high and could protect from ROS toxicity. Under biotic stress, this ascorbate-glutathione interplay could serve to fine-tune the content of ROS (Bordenave et al. 2013). This may be necessary to initiate and regulate the downstream defence mechanisms mediated by ROS.

Within the context of a close relationship between ascorbate and glutathione, compensatory responses between AsA and GSH have been reported during some plant–pathogen interactions. In the susceptible interaction of *Eucalyptus sieberi* with *P. cinnamomi,* under intense oxidative stress, the increase in AsA content compensated for the decrease in GSH (Dempsey et al., 2012). Similarly, in the interaction of *B. cinerea* with common ice plant (*Mesembryanthemum crystallinum*), a model intermediate C3-CAM (Crassulacean Acid Metabolism) plant, the pathogen-induced changes in the AsA and GSH contents and APX activity indicate that some compensatory ascorbate-related mechanisms could exist to ensure efficient antioxidant protection in the case of glutathione deficiency and vice versa (Gabara et al. 2012). The antioxidative compensatory mechanisms involved in the regulation of ascorbate- and glutathione-dependent defence described for ice plant–*B. cinerea* interaction were not found in tomato infected with this fungal pathogen (Kuźniak and Skłodowska 2005b). The latter supports the observation that the antioxidants are not interchangeable and ascorbate and glutathione can affect

different aspects of plant response to pathogens. Ascorbic acid is involved in the synthesis of hydroxyproline-rich glycoproteins and hormones as well as in gene expression (Zhang 2013). GSH is likely to exert a more general role in regulation of gene expression (Noctor et al. 2012; Schnaubelt et al. 2015). Different patterns of *B. cinerea*-induced ascorbate- and glutathione-related changes found in the resistant (ice plant) and susceptible (tomato) interaction indicate that besides a general conceptual framework of the role of these antioxidants in plant defence against pathogens, it has some elements specifically tailored for individual pathosystems.

Although the involvement of ascorbate and glutathione in plant responses to pathogens has been unambiguously shown, underlying molecular mechanisms remain poorly characterized. Ascorbate and glutathione have been repeatedly reported to play a complex role in plant resistance which is far beyond the ROS detoxification activity (Zhang 2013; Noctor et al. 2012). For example, early and high-dose ascorbate treatment as well as artificial glutathione enhancement by L-2-oxothiazolidine-4-carboxylic acid application alleviated the symptoms of virus infection in plants, whereas ROS eliminators such as dimethylthiourea and tiron, did not (Zechmann et al. 2007; Wang et al. 2011). These results point to a signalling role of these antioxidants in plant resistance to pathogens, also reported in other studies. The antioxidant capacity and redox state of the ascorbate and glutathione pools have been shown to regulate defence responses such as induction of PR proteins and phytoalexins (De Gara et al. 2003; Pastori et al. 2003). The ROS-antioxidant perturbations can initiate signalling pathways leading to further changes in the hormonal balance and gene expression (Foyer and Noctor 2011; Denancé et al. 2013). In this context, the AsA-GSH cycle is an antioxidant pathway but it also regulates the signalling potential of ascorbate and glutathione.

Studies on the interactions of glutathione-deficient *pad2* and *cad2* mutants of *Arabidopsis* with pathogens revealed that a certain level of glutathione, specific for a given pathosystem, is required for resistance (Ball et al. 2004; Parisy et al. 2007). The link between GSH synthesis and defence against pathogens was supported by the fact that the *Arabidopsis pad2* mutant containing only 30% of the wild type glutathione and decreased indole phytoalexin, camalexin level, was impaired in resistance to *P. brassicae* and *P. syringae* and lost the ability to induce HR response. The *pad2* mutation is localized in the GLUTAMATE-CYSTEINE LIGASE gene coding for the first enzyme of glutathione biosynthesis (Parisy et al. 2007). As the *pad2* mutant was also hyper-susceptible to other pathogens, these results point to the importance of glutathione in disease resistance of *Arabidopsis*. The glutathione-deficient *pad2 Arabidopsis* mutant also showed decreased insect resistance due to reduced accumulation of glucosinolates (Schlaeppi et al. 2008). In this mutant, the mechanisms of chemical defence were ineffective because the biosynthesis of camalexin and glucosinolates depends on glutathione acting as a S atom donor (Parisy et al. 2007; Schlaeppi et al. 2008).

Surprisingly, ascorbate deficiency has been shown to have opposite effects on plant resistance to pathogens. Ascorbate-deficient *Arabidopsis* mutants showed increased SA level and enhanced resistance to *P. syringae* whereas at high ascorbate

content the expression of PR genes was suppressed (Pastori et al. 2003; Pavet et al. 2005). These data suggest that ascorbate and glutathione, although tightly linked in the defence system, could have some specific functions in plant disease resistance to pathogens.

4 The Role of Compartment-Specific Changes in the AsA-GSH Cycle Activity in Biotic Stress Signalling

Biotic stress results in compartment-dependent ROS and redox signatures that could determine the specificity of plant response. In plant cells, each compartment contains its own set of ROS-generating and ROS-processing mechanisms; thus, the ROS and redox state of each compartment are maintained at different levels in accordance with its specific metabolic requirements. This gives rise to unique ROS and redox signatures generated under stress at the different compartments of the cell which contribute to the specific functions attributed to ROS and redox signals (Vanacker et al. 1998; Kuźniak and Skłodowska 2004a; Kuźniak and Skłodowska 2005a; Großkinsky et al. 2012).

The outcome of ROS-mediated signalling depends not only on the ROS signature but also on the site of ROS production. The interaction between ROS and related redox signals generated in different compartments is essential for stress signalling. Although considerable advances have recently been made concerning the ROS/redox signalling (Heyneke et al. 2013; Luschin-Ebengreuth and Zechmann 2016), the compartment-specific role of the AsA-GSH cycle during plant–pathogen interaction are still a matter of debate.

The oxidative effects of infection have been proved to affect all organelles, so the compartment-specific changes of ROS production and activity of the antioxidant system could be valuable indicators of stress (Kuźniak and Skłodowska 2005b). As these changes are masked when whole-cell extracts are analysed, their biological relevance might be underestimated. Observations on crude tissue extracts may provide only partial insight into the redox-dependent regulations of plant defence responses. The coordination of many ROS/redox signalling pathways between cellular compartments is necessary for maintaining energy and metabolic fluxes as well as for activating an adequate defence response (Fig. 1).

The apoplast is the first plant compartment where plant–pathogen interactions occur. It is therefore essential for pathogen recognition and the establishment of specific defence response (Qi et al. 2017). Apoplast is the major site of rapid ROS generation in plants infected by pathogens, the so-called oxidative burst. ROS produced mainly by RBOH, cell wall peroxidases and amine oxidases are released into the apoplast. At this location, they can directly affect the invading pathogen, serve as substrates in oxidative cross-linking of lignin precursors and of cell wall proteins, and regulate callose deposition at the cell wall, all being important features of plant resistance to pathogens (Bolwell et al. 2001; Li et al. 2017).

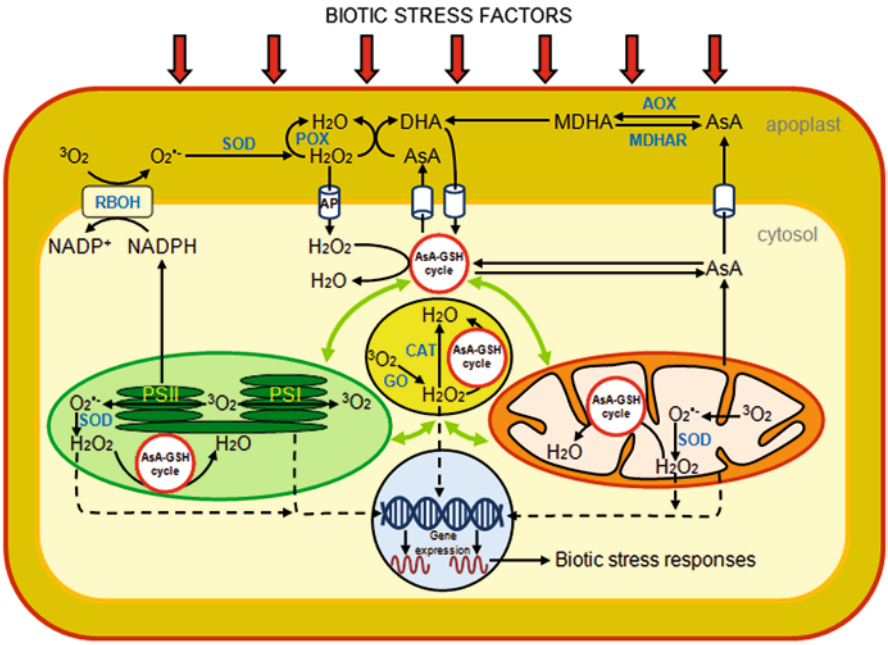

Fig. 1 Compartmentation of the ascorbate-glutathione (AsA-GSH) cycle in the plant cell and its role in biotic stress response. The AsA-GSH cycle is localized in several compartments of the plant cell, e.g. chloroplasts, mitochondria, peroxisomes, and the cytosol. The co-ordinated work of the cycle in different cellular compartments modulates the level of ROS, preventing cellular damage and regulating ROS signalling. Upon pathogen attack, O_2^- produced in the apoplast by the action of RBOH is converted by SOD to H_2O_2 which can be metabolized in the apoplast space either enzymatically by POX or non-enzymatically by AsA, or transmitted through the AP into the cytosol. The apoplastic H_2O_2- and ascorbate-dependent redox signals interfere with the AsA-GSH cycle components in other cellular compartments. Moreover, biotic stress induces disturbances in the electron fluxes in chloroplasts and mitochondria as well as in the peroxisomal metabolism leading to overproduction of H_2O_2 which is scavenged in the AsA-GSH cycle in these organelles or diffuses to the cytosol. Redox imbalances which occur in chloroplasts and mitochondria could participate to retrograde signalling. The signalling effects of H_2O_2 can be transduced by an increase in H_2O_2 itself and by perception of perturbed redox state via redox-sensitive proteins. It results in redox-dependent reprogramming of gene expression which shapes the plant defence response. *AsA* ascorbic acid (reduced), *AOX* ascorbate oxidase, *AP* aquaporin, *CAT* catalase, *DHA* dehydroascorbate, *GO* glycolate oxidase, *MDHA* monodehydroascorbate, *MDHAR* monodehydroascorbate reductase, *POX* cell wall peroxidase, *PSI/II* photosystems I and II, *RBOH* the respiratory burst NADPH oxidase homologue, *SOD* superoxide dismutase

Moreover, the apoplast O_2^-/H_2O_2 producing and scavenging mechanisms contribute to hormone-mediated stomata immunity (Baxter et al. 2014; Xia et al. 2015). Stomata function as part of the plant defence mechanisms against pathogens (Arnaud and Hwang 2015). Many bacteria and fungi with different lifestyles enter plants only through stomata while they are open, thus stomata closure prevents penetration through these pores and the subsequent colonization of the host tissues. Upon contact with pathogens and the perception of pathogen-associated molecular

patterns (PAMPs), plants activate a signalling cascade mediated by ROS and phytohormones, i.e. ABA and SA, which triggers stomata closure. Conversely, pathogens have evolved virulence factors which inhibit stomata closure or induce stomata reopening to counteract stomatal immunity. Some pathogens, e.g. *P. syringae*, can modify ABA signalling in plants and this hormone functions as a virulence factor for them (de Torres-Zabala et al. 2007). Interestingly, ABA and SA induce stomata closure through different ROS pools generated in the apoplast. ABA exerts its role via ROS produced by RBOH, whereas SA signalling is mediated by ROS generated by the cell wall peroxidases (Acharya and Assmann 2009; Miura et al. 2013).

Enzymatic and non-enzymatic AsA oxidation is an essential factor creating the apoplast redox status (Paciolla et al. 2016) and contributing to redox stress signalling. A potentially important role in the regulation of stomata aperture and stress signalling was suggested for the apoplastic DHA. In the leaf apoplast of tobacco plants overexpressing ascorbate oxidase, DHA resulting from oxidation of AsA that typically occurs under stress, has been reported to elicit stomata closure (Fotopoulos et al. 2008). These results emphasize the role of apoplastic AsA in the perception of environmental factors and the function of DHA as a potential stress signalling agent which can modulate plant responses to stress. The latter could be related to the ability of DHA to directly interact with GSH and with regulatory proteins containing cysteine residues (Morell et al. 1997). The ROS-induced modifications of these proteins are of physiological importance in regulating both plant metabolism and gene expression under stress (Dietz 2008; Gadjev et al. 2006).

The apoplastic ROS signals can also be transmitted across the plasma membrane and sensed in the cytoplasm, chloroplasts, and the nucleus resulting in changes in gene expression (Fig. 1). H_2O_2 can either enter the cell through specialized aquaporins, peroxiporins (Bienert et al. 2007; Hooijmaijers et al. 2012; Tian et al. 2016), or react with extracellular/transmembrane redox-sensitive proteins (Wrzaczek et al. 2010). Antioxidants located in the apoplast determine the lifetime and specificity of apoplastic ROS signalling. Oxidation of the apoplastic ascorbate and glutathione pools can also be involved in transmitting the apoplast-born redox signals to the cytosol and chloroplasts (Foyer and Noctor 2011). The redox states of ascorbate and glutathione redox couples provide information for acclimation and defence at the gene-expression level (Mou et al. 2003; Pastori et al. 2003; Zhang 2013).

In plant cells, the role of chloroplasts is far beyond the photosynthetic function of these organelles, as they are also involved in many other processes such as nitrogen and sulphur assimilation, production of secondary metabolites and phytohormones. Chloroplasts are sensitive sensors of environmental changes and their redox balance is easily perturbed (Kopczewski and Kuźniak 2013). The apoplastic ROS signal is transmitted via cytosolic signalling pathways to the chloroplasts, where a secondary ROS generation could be initiated (Shapiguzov et al. 2012). The accumulation of apoplastic H_2O_2 is also involved in the induction of the chloroplastic and cytosolic antioxidant enzymes, APX and GR (Hu et al. 2005). In the photosynthesizing plant cells, chloroplasts are the major sites of ROS production. They are, simultaneously equipped with complex antioxi-

dant systems, of which the AsA-GSH cycle has been most extensively studied (Asada 1999; Foyer and Noctor 2009). The chloroplasts in mesophyll cells contain 20–40% of the ascorbate and 10–50% of the leaf glutathione (Foyer and Noctor 2009). It has also been estimated that 65% of the total leaf DHAR activity and 70% of the total GR activity were localized in the chloroplasts of the mesophyll cells (Gillham and Dodge 1986). Changes in the chloroplast antioxidant system are part of the signalling pathways responsible not only for the optimization of photosynthesis and other metabolic processes, but also for stress signalling (Foyer and Noctor 2009). Redox signals arising from chloroplasts make an important contribution to immunity towards pathogens (Han et al. 2013). It has been suggested that enhanced H_2O_2 production in the light reflects a transient inactivation of the antioxidant system rather than an increased H_2O_2 production, possibly to trigger specific ROS-dependent defence responses (Trotta et al. 2014). This mechanism was likely to operate in chloroplasts of tomato leaves infected with *B. cinerea*, as the activity of APX and the contents of AsA and GSH were decreased by the pathogen. This was accompanied by the decrease in the ascorbate and glutathione redox ratios (Fig. 2, Kuźniak and Skłodowska 2001). Interestingly, the main H_2O_2 scavengers in the chloroplasts, APX, AsA, and GSH were down-regulated by the necrotrophic fungal pathogen and not by the biotrophic *P. syringae* pv *lachrymans*. In cucumber chloroplasts, the activity of APX and the content of AsA were only transiently decreased after *P. syringae* pv *lach-*

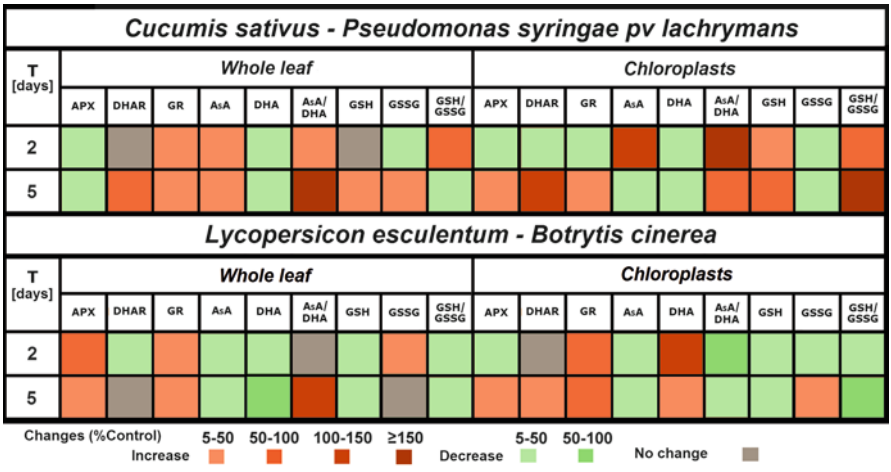

Fig. 2 Changes in the ascorbate-glutathione cycle activity in chloroplasts and whole-leaf extracts of cucumber (*Cucumis sativus*) infected with *Pseudomonas syringae* pv *lachrymans* and tomato (*Lycopersicon esculentum*) infected with *Botrytis cinerea.* Analyses were performed in whole-leaf extracts and chloroplasts 2 and 5 days after infection. The figure was drawn using mean values from control (non-infected) plants as 100% (Kuźniak and Skłodowska 1999, 2001; Kuźniak et al. 2016; Kopczewski, unpublished data). *AsA* ascorbic acid (reduced), *APX* ascorbate peroxidase, *DHA* dehydroascorbate, *DHAR* dehydroascorbate reductase, *GR* glutathione reductase, *GSH* glutathione (reduced), *GSSG* glutathione disulphide

rymans infection whereas the GSH content and the ascorbate and glutathione redox ratios were significantly increased (Fig. 2; Kuźniak et al. 2016). The uncoupling of the ascorbate- and glutathione-related redox system could reflect the specificity of the AsA-GSH cycle functioning in different compartments. The model study of Polle (2001) showed redox uncoupling of these two antioxidant pools in chloroplasts due to low rates of DHA formation owing to high MDHAR activity. In general, these results confirm that the AsA-GSH cycle-related antioxidant machinery in chloroplasts is targeted during pathogenesis although the pathogen-induced oxidative stress is generated mainly extracellularly, and the effect depends on the nature of pathogen. As biotrophs colonize the apoplastic space and necrotrophs immediately enter the plant cell, the diversity of responses could be related to the mode of action of the necrotrophic and biotrophic pathogens and based on how defence against these pathogens is controlled (Glazebrook 2005). For example, the defence mechanisms related to RBOH-mediated ROS production in the apoplast seem to be preferentially effective against biotrophic pathogens as *Arabidopsis rboh* mutants were resistant to the necrotrophic *Alternaria brassicicola* (Pogány et al. 2009).

The involvement of chloroplasts in plant immunity is further supported by the observation that the pathogen resistance of plants differs between light and dark, and in plants resisting pathogen attack the development of the HR requires light (Kuźniak et al. 2010). The fact that functional chloroplasts are required for the HR (Mateo et al. 2004) links plant immunity with light perception through their metabolism, including the chloroplastic AsA-GSH cycle. For example, chloroplast ROS and redox status were essential for the progress of HR during tobacco–*Xanthomonas campestris* pv *vesicatoria* interaction, but did not contribute to the induction of pathogenesis-related genes (Zurbriggen et al. 2009). The contribution of chloroplasts to plant immunity is broadened by hosting the biosynthetic pathways of SA and jasmonic acid which could regulate defence gene expression (Kangasjärvi et al. 2012). In the interaction of *B. cinerea* with common ice plant, the co-regulation of photosynthesis, antioxidant defence and immunity was also dependent on the metabolic type of photosynthesis. An infection-induced decrease in photochemical activity was found only in plants performing CAM metabolism (Gabara et al. 2012) and shortly after inoculation the activity of antioxidant enzymes was significantly affected by the redox state of plastoquinone (Nosek et al. 2015). Considering the timing of these changes, the results suggest that plants induced the resistant response in the form of HR-like lesions only when the redox state of the plastoquinone pool signalled high light conditions. Light and the redox state of photosynthetic electron transport were shown to play an important role in the conversion of L-galactono-1,4-lactone to AsA in the mitochondria, which determines the rate of AsA synthesis and influences its cellular level (Yabuta et al. 2008). Moreover, in CAM plants the steady-state and infection-induced AsA content and APX activity were significantly higher than in C3 plants, indicating an increased ascorbate-related antioxidant potential. A reverse relationship occurred for GSH which was higher in C3 plants (Gabara et al. 2012). Generally, these studies indicate that in C3 and CAM plants of *M. crystallinum*, distinct mechanisms were involved in the successful defence

against *B. cinerea* infection. They include specific co-regulation of the immune response, the photochemical activity of photosystem II and the antioxidant capacity. The activity of the AsA-GSH cycle in chloroplasts, the main regulator of the H_2O_2 content in these organelles, could also be implicated in the regulation of defence gene expression via the chloroplast-to-nucleus signalling (Fig. 1; Blanco et al. 2014).

As to the coordination of the ascorbate- and glutathione-dependent antioxidant defence in different compartments, it is worth noting that the AsA-GSH cycle enzymes, APX, MDHAR, and GR, are dual targeted to chloroplasts and mitochondria. Thus, the AsA-GSH cycle-dependent antioxidant defence is interrelated already at the gene level (Chew et al. 2003). Moreover, APX, MDHAR, and GR are also targets for post-translational modifications. They were differentially modified by nitration and S-nitrosylation mediated by NO-derived molecules. These processes led to irreversible inhibition and increase of cytosolic APX activity, respectively. The peroxisomal MDHAR was inhibited by these post-translational modifications whereas the chloroplastic and cytosolic GR were not affected (Begara-Morales et al. 2016). The complex interrelationship between cellular compartments was also exemplified by the study of Davletova et al. (2005), showing that in the absence of the cytosolic APX1, the chloroplast H_2O_2-scavenging system in *Arabidopsis* plants collapsed, leading to H_2O_2 accumulation and protein oxidation. Moreover, transgenic tobacco plants with suppressed cytosolic APX1 were hyperresponsive to pathogen infection, activating PCD in response to lower pathogen load than control plants (Mittler et al. 1999).

Although the diffusion of H_2O_2 from chloroplasts has been demonstrated in vitro (Mubarakshina et al. 2010) and aquaporins in the chloroplast envelope have been suggested to be involved in this process (Borisova et al. 2012), it seems that H_2O_2 itself is not the signal triggering nuclear gene expression. It rather acts through compartment-specific redox-sensitive elements which can transmit the signal to the nucleus (Petrov and Van Breusegem 2012; Kopczewski and Kuźniak 2013). In plants, hundreds of redox-target proteins with regulative functions have been identified, including transcription factors (Dietz 2008; Gadjev et al. 2006). This transmission, however, may be hindered by the redox buffering capacity of the cytoplasm. In tomato leaf cells infected with *B. cinerea*, the redox state of the cytoplasm was maintained on a significantly higher level than in the remaining cellular compartments. The cytoplasm was also better protected by the AsA-GSH cycle from the pathogen-induced oxidative stress than mitochondria and peroxisomes (Kuźniak and Skłodowska 2005b). This was proposed to be related to the necessity to withstand massive influx of DHA and GSSG from the apoplast during the oxidative burst (Horemans et al. 2000; Foyer et al. 2001).

Recently, a new scenario has been proposed according to which the fluctuating environmental conditions usually change the redox balance and induce moderate metabolic changes in the chloroplasts, signalled out of these organelles for acclimation, but ROS are kept under the intraorganellar control. However, under extreme stress, ROS accumulate to high levels, the chloroplast loses its integrity and H_2O_2 is released to the cytosol where it feeds into the MAPK (Mitogen-Activated

Protein Kinase) signalling pathway which controls defence gene expression and the HR (Dietz et al. 2016). The latter mechanism is likely to be activated in plants infected by pathogens or exposed to a combined action of abiotic and biotic stressors, which enhance lipid peroxidation leading to membrane permeabilization (Chojak et al. 2012).

The ROS/AsA-GSH cycle interaction produces organelle-specific redox signature which, similarly to ROS themselves, is critical for the retrograde signalling among different cellular compartments. This retrograde signalling has been extensively studied for chloroplasts and mitochondria (Blanco et al. 2014). Recently, it has been proposed that the WHIRLY1 protein perceives the redox changes in chloroplasts and is monomerized and translocated to the nucleus leading to acclimation and immunity responses. This mechanism, although analogous to the regulation of NPR1, could act as a NPR1-independent signalling pathway (Foyer et al. 2014).

A role in retrograde signalling has also been suggested for peroxisomes (Nyathi and Baker 2006; Corpas et al. 2017). In peroxisomes, which capacity to produce ROS is greater than in other compartments due to their essentially oxidative type of metabolism, the level of H_2O_2 is controlled by catalase and APX. However, under stress, which usually leads to overproduction of H_2O_2, it could be released into the cytosol through aquaporins in the peroxisomal membrane and initiate signalling cascades with different biological outcome (Corpas et al. 2017). Peroxisomal metabolism also results in generation of redox signals related to the ascorbate and glutathione redox state, as all components of the AsA-GSH cycle operate in these organelles (Kuźniak and Skłodowska 2005a; Nyathi and Baker 2006). In the tomato–*B. cinerea* interaction, the activities of all AsA-GSH cycle enzymes as well as ascorbate and glutathione concentrations and redox ratios in leaf peroxisomes were significantly decreased concomitant with disease symptom development. It indicated a collapse of the antioxidant protective system in peroxisomes at advanced stage of infection (Kuźniak and Skłodowska 2005a). At the early stage of fungal infection, however, peroxisomes preferentially accumulate at the sites where pathogen hyphae penetrate the plant cells (Koh et al. 2005). These results point to an important role of peroxisomes under infection, possibly related to the involvement of oxidative processes in preventing fungal penetration into plant epidermal cells (Mellersh et al. 2002).

Oxidative stress induced by infection triggers changes in the concentration and redox state of ascorbate and glutathione, but also in their subcellular distribution. In catalase-deficient *cat2 Arabidopsis* mutant exposed to oxidative stress, the concentration of GSSG in the vacuole increased from 5% to 25% of the cell glutathione. An enhanced accumulation of GSSG was also observed in chloroplasts where it may have consequences for redox-based reactions, including glutathionylation of thioredoxins (Queval et al. 2011; Noctor et al. 2013). The oxidation of GSH leading to the production of GSSG can be mediated by ROS and DHA. The reaction of GSH with H_2O_2 and O_2^- is slow whereas this with DHA occurs at a high rate and is accelerated by DHAR. Thus, it could be more important for the close collaboration of ascorbate and glutathione (Mhamdi et al. 2013). The

sequestration of GSSG in vacuoles and other compartments such as apoplast (Vanacker et al. 1998) and peroxisomes (Großkinsky et al. 2012) is an important element of the redox homeostasis and signalling through the glutathione-mediated system. Under control conditions, it serves to clear the cytosol of an active redox signal whereas under stress it helps to avoid excess stimulation and enables termination of stress signalling (Noctor et al. 2013).

The importance of the subcellular compartmentation in the AsA-GSH cycle-related antioxidant defence against pathogens has been evidenced in several plant–pathogen interactions (El-Zahaby et al. 1995; Vanacker et al. 1998; Kuźniak and Skłodowska 2005b). However, the patterns of spatiotemporal changes in the AsA-GSH cycle activity seem to be related to the plant–pathogen interaction to ensure an adequate defence response (Fig. 2; Großkinsky et al. 2012; Kuźniak and Skłodowska 2005b). The up-regulation of the AsA-GSH cycle, seen as plant's general response to oxidative stress imposed by pathogens is accompanied by diverse responses specifically tailored to a given host–pathogen interaction and related rather to its functions in multiple nodes of the signalling network. In the tomato–*B. cinerea* pathosystem, a general shift of the cellular redox balance towards oxidative state was found in the whole-leaf extracts as well as in chloroplasts, mitochondria, and peroxisomes (Kuźniak and Skłodowska 2005b). The timing and intensity of changes were compartment dependent and the pro-oxidative effect was more pronounced in peroxisomes and mitochondria when compared with chloroplasts and the cytosol. It was manifested by the decrease in the contents and redox ratios of the ascorbate and glutathione pools as well as by the insufficient activities of MDHAR, DHAR, and GR, reductases required for the recycling of AsA and GSH. This indicated that the cellular compartments were differentially protected by the AsA-GSH cycle. These compartment-specific changes could have implications for the redox signalling and the coordination of defence mechanisms (Kopczewski and Kuźniak 2013).

5 The Ascorbate- and Glutathione-Dependent Mechanisms in Plant Response to Abiotic and Biotic Stress Combinations

Among multiple combinations of environmental stress factors which can affect plants under natural conditions, those between abiotic stresses and pathogens have been shown to be important growth- and yield-limiting interactions (Mittler 2006; Suzuki et al. 2014). The plant response to stress combination is dependent on the type of stress factors, the initial threshold of stress, and the individual plant's stress history (Rasmussen et al. 2013; Pandey et al. 2015). Many evidence from field and laboratory studies suggests that plants respond to a specific combination of stresses in a non-additive manner, showing changes on metabolic, physiological, and molecular levels which cannot be predicted from the knowledge of single stress effects (Atkinson and Urwin 2012). For example, under combined stress conditions, changes of the levels of ROS, the expression of ROS-processing

enzymes and antioxidants differ from those induced by stress factors applied individually (Sewelam et al. 2016). Maintaining the redox balance is critical for the normal plant growth and performance (Ellouzi et al. 2014) and tight coordination of ROS production and processing mechanisms during combined biotic and abiotic stress is particularly important. Transcriptional, quantitative genetic, and agronomic studies showed that plants respond to multiple stresses using similar defence signalling pathways mediated by ROS, redox signals, and phytohormones. ROS-mediated mechanisms, which involve disturbances in the cellular ROS homeostasis followed by changed detoxification capacity, link abiotic and biotic stress defence (Perez and Brown 2014).

Recently, the concept of priming has gained considerable attention as a potent mechanism of producing multiple stress-resistant crops. It is well known that after a mild, primary stress which promotes a plant to a "primed" state, it shows tolerance to a second, strong stress (Wiese et al. 2004; Conrath et al. 2015). This mechanism allows plants to adjust to multiple stresses. Shared transcriptional response and ROS signalling have been proposed to play a key role in inducing this process (Perez and Brown 2014), and there are several reports on the abiotic stress factors, mainly salinity and drought, affecting plant resistance to pathogens. Most of them indicate that abiotic stress compromised the defence response to pathogens (You et al. 2011; Chojak et al. 2012; Ramegowda and Senthil-Kumar 2015; Chojak-Koźniewska et al. 2017). However, there are no reports concerning the involvement of AsA-GSH cycle in plant tolerance to abiotic and biotic stresses acting in combination. The available reports refer mainly to the activity/concentration changes of some AsA-GSH cycle components in plants exposed to metal stress and infected. For example, Nenova and Bogoeva (2014) focused only on APX activity changes in response to copper and *Fusarium culmorum* in wheat leaves and roots. They found organ-specific and copper dose-dependent trend in decreasing of APX activity when copper and *F. culmorum* acted together, while in plants treated with copper individually APX activity was up-regulated. Another study revealed that Al treatment increased APX activity in *Cajanus cajan*, whereas Al in combination with *F. incarnatum* down-regulated it (Satapathy et al. 2012). In cucumber pre-treated with 100 mM NaCl for 7 days and then infected with *P. syringae* pv *lachrymans*, differences in timing and intensity of H_2O_2 and O_2^- accumulation between plants exposed to combined stress and to individual action of salinity and the pathogen were observed. Infection-induced oxidative stress was stronger when cucumber plants were pre-treated with NaCl, as shown not only by enhanced ROS accumulation but also by an increase in lipid peroxidation (Chojak et al. 2012). Moreover, salinity intensified disease symptoms on cucumber leaves, resulting in more extensive necrosis with chlorotic halos, and increased bacteria proliferation at the late stage of infection, compared to plants infected without NaCl pretreatment (Chojak-Koźniewska et al. 2017). This was accompanied by modifications of the antioxidant system response, exemplified by the induction of FeSOD, a chloroplastic SOD isoform found in cucumber plants under combined stress at early stage of pathogenesis (Chojak-Koźniewska et al. 2017). Activation of FeSOD indicated that enhanced antioxidant protection of these organelles was necessary under multiple

stress conditions (Mittler 2017). Moreover, H_2O_2 generated by FeSOD could impact the AsA-GSH cycle and SA signalling, as chloroplasts are also sites of SA synthesis which is essential in plant immunity to biotrophic pathogens (Dempsey et al. 2011; Suzuki 2016). In *Arabidopsis* mutants lacking FeSOD, down-regulation of chloroplastic genes and impaired chloroplast development was observed (Pilon et al. 2011). Thus, the increased activity of FeSOD could be important to sustain the role of chloroplasts in plant defence against pathogens.

The AsA-GSH cycle, which is a major component of the antioxidant system, is also one of the most important mechanisms controlling oxidative balance of the plant cell under stress (Foyer and Noctor 2011). Mutants impaired in the AsA-GSH cycle showed high ROS levels and increased sensitivity to stress (Huang et al. 2005). The AsA-GSH cycle is integrated in response to multiple stress by transcription factors. In *Arabidopsis*, zinc-finger transcription factor ZAT12, which responses to high H_2O_2 content, is induced by several abiotic and biotic stress factors. It induces the expression of APX genes resulting in tolerance to oxidative stress (Atkinson and Urwin 2012).

In cucumber plants, all AsA-GSH cycle components were affected under combined salt and biotic stress (Fig. 3). This combination strongly modified the antioxidant system response compared to individual stressors, indicating that plant cells exposed to a combination of abiotic and biotic stresses require specific antioxidant protection. In cucumber leaves under combined stress, APX activity was significantly decreased compared to plants infected with the bacterial pathogen without exposition to NaCl (Fig. 3). Changes in APX activity are common stress markers (AbdElgawad et al. 2016). Moreover, APX, especially its cytosolic and chloroplastic isoforms, is a key element of stress signalling (Shigeoka et al. 2002). The decreased APX activity, besides being the effect of AsA depletion, could be related to SA signalling, as one of the proposed models of SA action is the inhibition of APX (Durner and Klessig 1995) and SA biosynthesis takes place in chloroplasts (Dempsey et al. 2011).

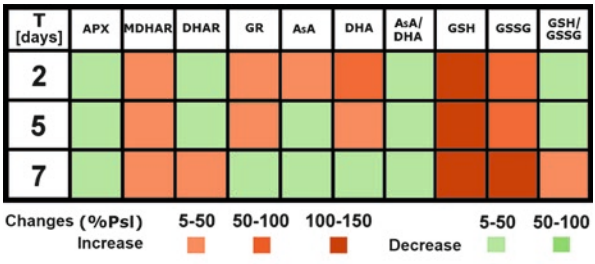

Fig. 3 The effect of NaCl pretreatment on the ascorbate-glutathione cycle activity in leaves of cucumber plants (*Cucumis sativus*) after *Pseudomonas syringae* pv *lachrymans* infection. Cucumber plants were pre-treated for 7 days with 100 mM NaCl and then infected with *P. syringae* pv *lachrymans*. Analyses were performed 2, 5, and 7 days after inoculation. The figure was drawn using mean values from plants infected without salt treatment as 100% (Chojak-Koźniewska 2017). *AsA* ascorbic acid (reduced), *APX* ascorbate peroxidase, *DHA* dehydroascorbate, *DHAR* dehydroascorbate reductase, *GR* glutathione reductase, *GSH* glutathione (reduced), *GSSG* glutathione disulphide, *MDHAR* monodehydroascorbate reductase

The activity of DHAR was mostly down-regulated which indicates lowered capacity to regenerate AsA under combined stress, as confirmed by DHA accumulation. MDHAR activity was increased suggesting that AsA regeneration is mediated mainly by this enzyme, but only at the early stage of infection (Chojak-Koźniewska 2017). Results for cultivated tomato *Lycopersicon esculentum* (*Solanum lycopersicum*) and its wild salt-tolerant relative *L. pennellii* confirmed the role of MDHAR in AsA regeneration (Mittova et al. 2000). However, at the advanced stage of pathogenesis in cucumber plants pre-treated with NaCl, ascorbate recycling via MDHAR appeared to be ineffective as AsA content decreased. The decreased APX activity under combined stress, accompanied by increased ROS generation and lipid peroxidation as well as lowered AsA content and DHA accumulation, indicated an enhanced oxidative burden imposed by multiple stress (Fig. 3; Chojak et al. 2012; Chojak-Koźniewska et al. 2017). Salt stress and *Pseudoperonospora cubensis* infection applied sequentially on cucumber plants induced oxidative stress and increased the activities of APX and GR, but it was insufficient enough for plants to be protected from oxidative stress (Nostar et al. 2013).

In cucumber leaves, when NaCl and the pathogen acted together, the activity of GR increased at the early stage of infection, compared to the infected plants (Fig. 3). It could indicate an increased demand for GSH being a key signalling molecule under biotic stress, at the time points when plant defence mechanisms may be mobilized (Noctor et al. 2012). Changes in the AsA-GSH cycle enzyme activities observed in cucumber (Fig. 3) indicated enhanced cycling of AsA and GSH which may contribute to the coordination of multifactorial response.

Changes in the total concentration of ascorbate and glutathione, their redox forms as well as changes in AsA/DHA and GSH/GSSH redox ratios under stress conditions provide information about cell redox homeostasis which is crucial for cell metabolism regulation, growth processes, and activation of plant defence responses (Zechmann 2014; Noctor and Foyer 2016). Increasing the contents of AsA and GSH through ascorbate/glutathione recycling has been found to be advantageous for plants under stress because it can limit the deleterious effects of oxidative stress and reset the redox homeostasis. Changes in the AsA and GSH pools in cucumber leaves occurred both after bacterial infection acting alone (data not shown) and in combination with NaCl and were dependent on the stage of infection development. Under combined stress, however, the tendency to decrease AsA and DHA accumulation compared to the plants infected without NaCl pretreatment prevailed. Moreover, NaCl intensified the post-infection accumulation of GSH and GSSG in the leaves (Fig. 3). Increased GSH content, not accompanied by an increase in AsA, may indicate a substantial change in the intracellular redox state. The changes in the ascorbate pool could have important consequences for plant response to multiple stress as AsA serves as the cofactor for enzymes involved in ABA biosynthesis, is involved in phytohormone-mediated signalling and low ascorbate content enhances SA and ABA signalling pathways (Pastori et al. 2003). According to Foyer and Noctor (2011), changes in AsA and DHA levels are difficult to unambiguous interpretation because much of the DHA pool is stored in apoplast and spatially separated from the ascorbate intracellular pool. It is therefore possible

that the overall intracellular ascorbate pool is maintained in a reduced state, even during oxidative stress, due to the redox buffer capacity of the cytosol. The cytosolic environment in the photosynthesizing plant cells is usually highly reducing due to a significant excess of the reduced over the oxidized forms of ascorbate and glutathione (Fedoroff 2006). Moreover, maintaining the low apoplast AsA/DHA redox ratio is extremely important for stomatal movement control and response to stress (Chen and Gallie 2004). In cucumber plants treated with NaCl and infected with *P. syringae* pv *lachrymans* (Fig. 3), changes in ascorbate and glutathione pools were nonparallel. The uncoupling of these redox pairs could be due to limitations in DHAR activity (Fig. 3). In cucumber plants exposed to salt stress and then infected, the AsA/DHA redox ratio decreased compared to plants infected without NaCl treatment, whereas the GSH/GSSG ratio was increased at the late stage of pathogenesis. Moreover, changes in AsA/DHA and GSH/GSSH were much stronger than in cucumber plants treated with these stressors individually (Chojak-Koźniewska 2017). These results support the concept that AsA and GSH perform specific functions in plant cells and should not be regarded as equally valued antioxidants.

The regeneration of AsA and GSH via the AsA-GSH cycle depends on NADPH which supply can be limited under stress. The acclimatization to abiotic stresses and defence reactions to pathogens, which require energy inputs and diversion of carbon metabolites to anabolic pathways, are associated with reprogramming of the primary metabolism. Thus, the NADPH generating dehydrogenases, such as NADP-malic enzyme (NADP-ME) and NADP-isocitrate dehydrogenase (NADP-ICDH) could be important to support the AsA-GSH cycle action under stress (Noctor 2006; Leterrier et al. 2012). In *Fragaria vesca*, NADP-ME has been shown to deliver NADPH for regenerating GSH via GR action (Blanch et al. 2013). NADPH production via NADP-ICDH has been shown to be important in promoting glutathione-dependent redox signalling and maintaining redox stability in response to post-infection oxidative stress (Mhamdi et al. 2010).

The reaction of tomato plants exposed to salt stress and *P. syringae* pv *lachrymans* infection confirmed that combination stress generates unique ROS and AsA-GSH cycle-related redox signatures (Fig. 3). The response to the combined stress resulted in novel interactions between the AsA-GSH cycle components, especially ascorbate and glutathione. This implies specific adjustments of the AsA-GSH cycle components to other elements of the stress signalling network, e.g. ROS and phytohormones such as SA or ABA (Mhamdi et al. 2013; Baxter et al. 2014; Xia et al. 2015). ROS and SA act in a regulatory loop wherein ROS induce SA accumulation and SA enhances their production which finally activate antioxidants (Khan et al. 2015). Combined stress changed the SA/ABA balance which hindered the PR1 gene expression, known to be regulated by the glutathione redox status (Noctor et al. 2012). Salt stress through ABA up-regulation could have antagonistic effects on SA-mediated signalling and compromises the defence against *P. syringae* pv *lachrymans*, a biotrophic pathogen (Chojak-Koźniewska et al. 2017). In the interplay between ABA, SA, and ROS signalling pathways, ABA has been suggested to be positioned between ROS and SA (Kissoudis et al. 2014).

6 Conclusions

Biotic stressors affect the redox balance of plant cells and the AsA-GSH cycle has an important role in managing the ROS generated in response to pathogens. In addition to its direct involvement in ROS processing, the AsA-GSH cycle may protect against biotic stress by activating defence mechanisms through redox signalling, and ascorbate and glutathione seem to fulfil a central role in this process. Ascorbate and glutathione are the key players in redox regulatory systems and they function in multiple nodes of the defence signalling network. The versatile cross-communication of glutathione and ascorbate with other signalling molecules in the plant defence network is crucial for conditioning the response to abiotic and biotic stress combinations which are common to many agricultural areas. At the subcellular level, the interplay between ROS and the AsA-GSH cycle components generates compartment-specific stress response signature which plays important role in sensing, signalling, and activating plant defence.

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