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REVIEW PAPER

The ascorbate–glutathione cycle coming of age

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Abstract

Concepts regarding the operation of the ascorbate–glutathione cycle and the associated water/water cycle in the processing of metabolically generated hydrogen peroxide and other forms of reactive oxygen species (ROS) are well established in the literature. However, our knowledge of the functions of these cycles and their component enzymes continues to grow and evolve. Recent insights include participation in the intrinsic environmental and developmental signalling pathways that regulate plant growth, development, and defence. In addition to ROS processing, the enzymes of the two cycles not only support the functions of ascorbate and glutathione, they also have 'moonlighting' functions. They are subject to post-translational modifications and have an extensive interactome, particularly with other signalling proteins. In this assessment of current knowledge, we highlight the central position of the ascorbate–glutathione cycle in the network of cellular redox systems that underpin the energy-sensitive communication within the different cellular compartments and integrate plant signalling pathways.

Keywords: Foyer–Halliwell–Asada cycle, hydrogen peroxide, photosynthesis, redox signalling, ROS wave, superoxide, stress acclimation.

Introduction

It is almost 50 years since the ascorbate–glutathione cycle (sometimes called the Asada–Halliwell–Foyer cycle or Foyer–Halliwell–Asada pathway; Fig. 1) was first described in chloroplasts (Foyer and Halliwell, 1976). This was together with the proposal that the function of this cycle was to protect redox-sensitive proteins from uncontrolled oxidation by reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2). Thereafter, soluble ascorbate-specific peroxidases (APXs) were described for the first time (Groden and Beck, 1979; Kelly and Latzko, 1979). Intensive biochemical and molecular/genetic research efforts in the following decades demonstrated that ascorbate, glutathione, and other components of this cycle can be found in every compartment of the plant cell (Noctor and Foyer, 1998). Although the importance of antioxidants, such as ascorbate and glutathione in human diseases, had long been recognized, it was only somewhat later that the interactions between ascorbate and glutathione were considered in animal systems (Meister, 1994). Ascorbate and glutathione are the most abundant low molecular weight (LMW) antioxidants in plant cells, and their primary functions are related to interactions with ROS and other reduction/oxidation- (redox) sensitive molecules. Together with peroxiredoxins (PRXs) and thioredoxins (TRXs), the ascorbate–glutathione cycle regulates ROS accumulation in each compartment, with perhaps

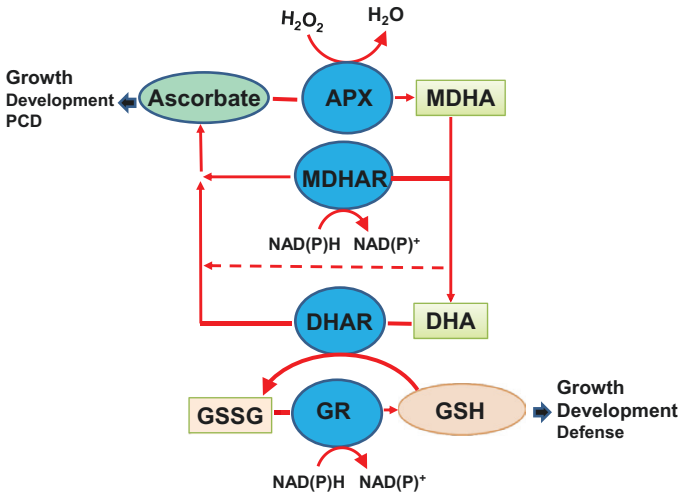

Fig. 1. The role of the ascorbate–glutathione cycle (Asada–Halliwell–Foyer cycle) in regenerating the reduced forms of ascorbate and glutathione to maintain a wide range of biological functions. APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; GSH, reduced glutathione: GR, glutathione reductase; GSSG, glutathione disulfide; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase.

the exception of the apoplast/cell wall compartment (Foyer and Hanke, 2022). These PRXs reduce not only H_2O_2 but also alkyl hydroperoxide and peroxynitrite (Liebthal *et al.*, 2018), regulating the concentration of cellular peroxides. However, ascorbate and glutathione are multifunctional metabolites with diverse interactomes that facilitate a wide range of functions in the regulation of plant growth and development as well as defence. Moreover, each of the four component enzymes, namely APX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), have subcellular isoforms that can serve different, sometimes 'moonlighting', functions that remain poorly characterized and understood. Hence, the action of the ascorbate–glutathione cycle extends far beyond policing ROS signals, not least because this pathway serves to maintain the reduced states of the ascorbate and glutathione pools that fulfil important but divergent roles in plant biology (Pellny *et al.*, 2009; Pasternak *et al.*, 2020; Zur *et al.*, 2021). The following discussion provides a current overview of the relevant literature, highlighting the regulation and functions of different components of the cycle, with a particular focus on signalling and regulation. We also consider possibilities for other additional functions related to the individual roles of ascorbate and glutathione.

ROS processing and regulation of ROS signalling

Accumulating evidence suggests that ROS are essential metabolite markers, or signals of living cells (Van Breusegem *et al.*, 2018). During evolution, the management of oxygen metabolism and the associated production, accumulation, and degradation of ROS in each extracellular and intracellular compartment has become central to every aspect of biology from energy metabolism to growth, development, and defence. Superoxide and H_2O_2 act as either electron donors (reductants) or acceptors (oxidants). They thus engage in electron transfer (redox) processes with cellular metabolites and proteins. ROS are an integral part of the cell decision-making process in all aerobic cells, and hence overaccumulation of ROS can lead to growth arrest and cell death. However, the notion that there are 'low' and 'high' levels of ROS in plant cells that have different functions is misleading because it suggests that low ROS levels are focused on signalling while high ROS levels are involved in more negative reactions rather than signalling. In fact, all ROS molecules are potentially effective signalling molecules; no matter the level of accumulation, the capacity for signalling is limited only by the availability of interacting partners that can transfer the oxidative signal. There is little evidence that oxidative damage accumulates in plant cells to such an extent that it limits cellular functions. In many cases, ROS and oxidized lipids and proteins also function as signals that regulate gene expression to ensure appropriate acclimation or cell death responses. ROS accumulation leading to an enhanced oxidative state is a key signature of plant responses to biotic and abiotic stresses such as drought, heat, salinity, and high light (Choudhury *et al.*, 2017). Moreover, many aspects of plant development, such as the maintenance of stem cells and quiescence, and seed germination, involve an imposed 'oxidative state', as discussed in detail below. Each subcellular compartment in plants contains its own set of ROS-producing and ROS-scavenging pathways, but relatively little is known about how the different components in such compartmentalized systems are coordinated. Choudhury *et al.* (2017) concluded that as long as plant cells maintain high enough energy reserves to remove ROS, these essential signals are beneficial to plants during abiotic stress, enabling them to adjust their metabolism and mount a proper acclimation response. The functions of the ascorbate–glutathione cycle are powered by the pools of pyridine nucleotides, NAD(H) and NADP(H), as is ROS production by respiratory burst oxidase homologues (RBOHs) and other ROS-producing enzymes. These essential co-enzymes function as energy transducers, signalling molecules, and redox couples, the balance between the oxidized and reduced forms being important in the maintenance of cellular redox status, regulation of ion channels, and responses to environmental and metabolic challenges that determine cell fate (Noctor *et al.*, 2006).

ROS signals fulfil important roles in the regulation of numerous developmental processes from root development (Eljebbawi *et al.*, 2021; Mase and Tsukagoshi, 2021), the transition to flowering (Huang *et al.*, 2021), to leaf senescence (Zentgraf *et al.*, 2022). They contribute to the elicitation of genetic and epigenetic responses that allow acclimation and adaptation to metabolic, developmental, and environmental triggers (Ramakrishnan *et al.*, 2022). ROS and the ascorbate–glutathione cycle thereby function synchronously to regulate plant growth and development, as well as defence. For example, ROS generation is the driver and first requirement for many

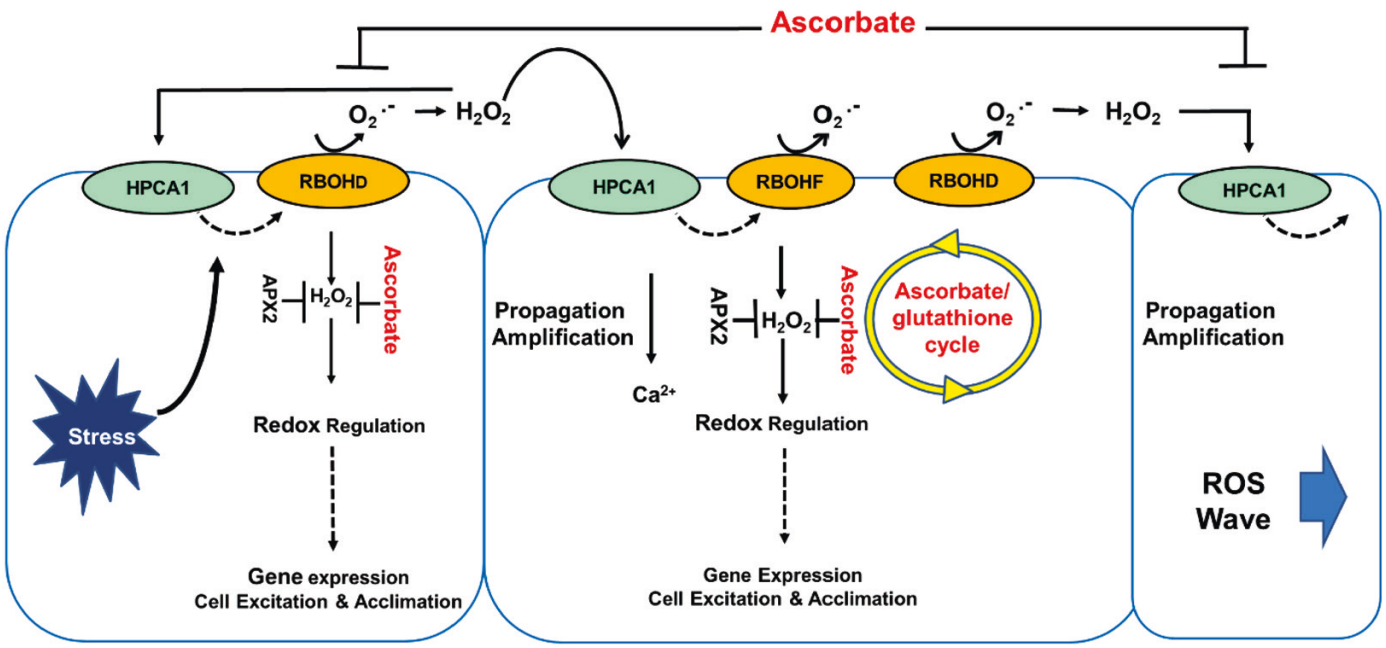

Fig. 2. The role of antioxidants in modulating the ROS wave pathway of systemic signalling. APX2, ascorbate peroxidase2; HPCA1, HYDROGEN-PEROXIDE-INDUCED CA^{2+} INCREASES (HPCA)1; RBOHD, F, respiratory burst homologue protein D, F in *Arabidopsis thaliana*.

developmental processes, such as the cell cycle, pollen viability, microspore reprogramming towards sporophytic development, the regulation of female gametophyte patterning, and the maintenance of embryo sac polarity, as well as the prevention of self-pollonation (de Simone *et al.*, 2017; Sankaranarayanan *et al.*, 2020; Zhang *et al.*, 2021; Zur *et al.*, 2021). In such systems, the ascorbate–glutathione cycle, together with TRXs, PRXs, glutaredoxins (GRXs), and antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), ensures appropriate redox-mediated regulation, so that ROS, ascorbate, and glutathione can accumulate in the required compartment-specific manner.

Cell-to-cell ROS signalling plays a pivotal role in activating local and systemic responses to environmental and developmental signals (Waszczak *et al.*, 2018; Zandalinas *et al.*, 2020, 2021; Fichman and Mittler, 2021a, b). Auto-propagating waves of ROS, calcium, and electric signals function together to generate rapid systemic cell-to-cell communication (Wang *et al.*, 2019). Succesive waves of ROS accumulation and removal are, therefore, important not only in cell-to-cell communication in plants (Zandalinas *et al.*, 2020, 2021; Fichman and Mittler, 2021a, b), but also for plant-to-plant communication (Szechyńska-Hebda *et al.*, 2022), plant–microorganism interactions (Zhou *et al.*, 2019), signalling between mammalian cells, and also in isolated animal hearts, allowing coordinated acclimation responses (Fichman *et al.*, 2023). The cell-to-cell transmission of the ROS wave can, however, be blocked by the addition of antioxidants, such as CAT or inhibitors of NADPH oxidase (also called RBOH proteins). This demonstrates that this system of cell-to-cell communication is policed by regulated production and destruction of ROS signals. The activation of RBOH proteins on the plasma membrane generates superoxide radicals in the apoplast, which are converted to H_2O_2, through either spontaneous dismutation or the action of SOD.

The process of cell-to-cell ROS signalling, which is called the 'ROS wave' (Fig. 2), is linked to cell-to-cell calcium and membrane potential signalling and is essential for systemic stress signalling and systemic acquired acclimation (Fichman and Mittler, 2020, 2021a, b). In this process, ROS production by the RBOHs, RBOHD and RBOHF, is triggered in the cells that are directly subjected to stress, resulting in a state of 'activated ROS production'. The leucine-rich repeat receptor-like kinase HPCA1 (H_2O_2-induced Ca^{2+} increases 1) is required for coordination of ROS and calcium signals during the cell-to-cell propagation of ROS signals (Fichmann *et al.*, 2022). Once the state of activated ROS production reaches cells and tissues, other than those initiating the signal, it triggers acclimation mechanisms and enhances overall stress resilience (Fichman and Mittler, 2021a, b). Little attention has as yet been paid to how the ascorbate/gluathione cycle regulates the lifetime of ROS signals in any given cellular compartment. ROS processing and removal in activated cells is, however, an essential feature of the progression of the ROS wave.

The ascorbate–glutathione-dependent water/water cycle and its functions

The ascorbate–glutathione cycle is comprised of metabolites (ascorbate, glutathione, and NADPH) and enzymes, which

regenerate the reduced forms of ascorbate and glutathione (Fig. 1). The first step of the pathway is the reduction of H_2O_2 to water by the action of APX, using ascorbate acting as the electron donor. Oxidized ascorbate (monodehydroascorbate, MDA) can thereafter either spontaneously disproportionate to ascorbate and dehydroascorbate (DHA), or be reduced to ascorbate by the enzyme MDHAR, using the reducing power of NAD(P)H. In addition, the photosynthetic electron transport chain may directly reduce MDHA to ascorbate (Miyake and Asada, 1994). DHA is reduced back to ascorbate by several enzyme systems, as discussed below. However, in the classic formulation of the ascorbate–glutathione pathway (Foyer and Halliwell, 1976), DHA is reduced to ascorbate by the enzyme DHAR using reduced glutathione (GSH) as the reductant. The enzyme GR then reduces the oxidized form of glutathione, glutathione disulfide (GSSG), to GSH with NADPH as the reductant (Foyer and Halliwell, 1976).

The water/water cycle (WWC) is a logical extension of the activity of the ascorbate–glutathione cycle in chloroplasts (Asada, 1999), because the production and removal of H_2O_2 to water is coupled to the activity of the photosynthetic electron transport (PET) chain (Foyer and Hanke, 2022; Fig. 3). The water-splitting activity of PSII facilitates the transfer of elections through the PET chain to produce reduced ferredoxin and NADPH, and also produces molecular oxygen. In turn, molecular oxygen can accept electrons from many of the electron carriers in the PET chain (Foyer and Hanke, 2022), a process that is called the 'Mehler reaction', or 'pseudocyclic electron flow'. The univalent reduction of oxygen by the PET chain produces superoxide ($O_2^{\cdot-}$) radicals, largely at the surfaces of the thylakoid membranes. Superoxide produced on the stromal surfaces of the membranes is then rapidly converted to H_2O_2 by the action of thylakoid SODs. Thereafter, H_2O_2 is reduced to water by chloroplast APXs and the ascorbate–glutathione cycle, and also by the action of 2-Cys peroxiredoxins (PRXs). They are re-reduced either by the chloroplast TRX system (Foyer and Hanke, 2022) or by GSH and GR. Taken together, these reactions form the WWC, in which two electrons are used to produce H_2O_2 and two more electrons are required to metabolize H_2O_2 to water (Fig. 3). The WWC ultimately provides a mechanism for the dissipation of excess excitation energy and electrons, in which molecular oxygen is used as an alternative electron sink. This pathway may provide protection of PSII from photoinhibition, which still supports ATP production (Neubauer and Yamamoto, 1992). The WWC also plays a role in regulating the oxidation state of the chloroplast-targeted 2-Cys PRXs, which, together with specific atypical TRXs such as ACHT1–ACHT4 and TRXL2, are involved in the transfer of oxidative equivalents from H_2O_2 to target chloroplast proteins, such as those of the reductive pentose pathway (Ojeda *et al.*, 2018; Vaseghi *et al.*, 2019; Yokochi *et al.*, 2021). Similarly, the WWC plays a role in the regulation of cyclic electron flow around PSI (CEF), which serves to balance the energy budget of photosynthesis (Strand *et al.*, 2015). In this system, H_2O_2 functions as a signal that activates the CEF pathway, while the ascorbate–glutathione pathway serves to modulate the signal. Likewise, the primary precursor of jasmonic acid (JA), 2-oxophytodienoic acid (OPDA), interacts with 2-Cys PRX, which is suggested to act as a redox sensor through H_2O_2 processing and associated regulation of the TRX- and thiol-dependent regulation of enzymes of the Benson/Calvin cycle such as fructose 1,6-bisphosphatase (FBPase; Muthuramalingam *et al.*, 2013; Liebthal *et al.*, 2018). OPDA also binds to cyclophilin 20-3 (CYP20-3), which forms a complex with serine acetyltransferase 1 (SAT1). This, in turn, triggers the formation of a hetero-oligomeric cysteine synthase complex (CSC) with *O*-acetylserine(thiol)lyase B, a process that activates sulfur assimilation and the accumulation of sulfur-containing metabolites such as GSH (Watanabe *et al.*, 2021).

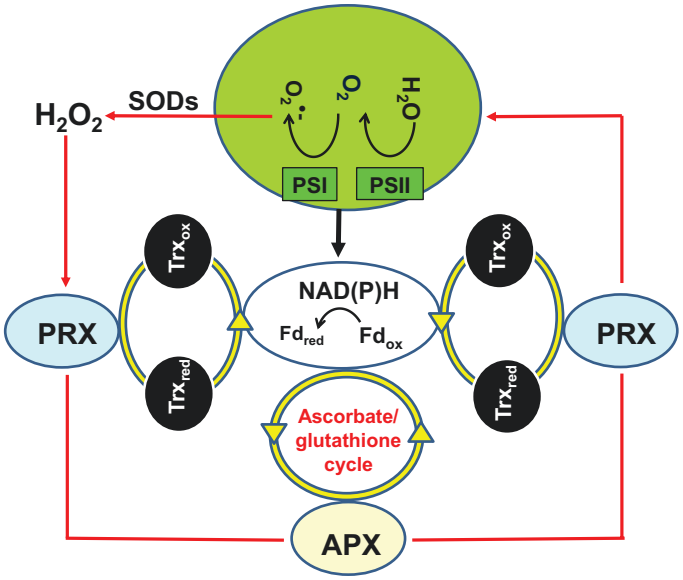

Fig. 3. The water/water cycle allowing the dissipation of excess excitation energy and electrons providing an alternative electron sink for protection of PSII from inhibition and supporting ATP production. APX, ascorbate peroxidase; Fd_{ox}, oxidized ferredoxin; Fd_{red}, reduced ferredoxin; $O_2^{\cdot-}$, superoxide anion radicals; PRX, 2-Cys peroxiredoxins; Trx_{ox}, oxidized thioredoxin; Trx_{red}, reduced thioredoxin.

The 2-Cys PRXs operate through the formation of a homodimer, in which a disulfide bond connects a peroxidatic Cys $(C^P)^{175}$ from one monomer and is connected to the resolving Cys located on the second monomer. The oxidation of $(C^P)^{175}$ inhibits 2-Cys PRX activity. Reduction of the 2-Cys PRX dimers requires reductants such as GSH, TRXs, NADPH-dependent TRX reductase C (NTRC), and/or CYP20-3 (Liebthal *et al.*, 2018). GSH binding regulates the conformational state of 2-Cys PRX, favouring monomerization (Liu *et al.*, 2020). This suggests that GSH is an effective reducing agent for 2-Cys PRX that regulates the roles and functions of this redoxin in chloroplasts. Like 2-Cys PRX, other PRXs can oxidize GSH via the action of

GRX (Rahantaniaina *et al.*, 2013). The active site Cys residues of Prx2 can form stable mixed disulfides with reduced GSH (Peskin *et al.*, 2016) in a glutathionylation reaction that is reversed by GRX1.

The light-dependent thiol-dependent regulation of enzymes, such as FBPase and the 46 kDa isoform of Rubisco activase, is essential for the efficient operation of photosynthesis and carbon assimilation, as well as carbon partitioning and transport. This central system of photosynthetic regulation is based on the redox modulation of Cys residues on proteins such as FBPase that have a high intrinsic reactivity. Cys residues have nucleophilic thiol side chains that are susceptible to oxidative modifications and are hence amongst the most reactive amino acids. The oxidation of Cys thiols leads to the formation of sulfenic acid (–RSOH), which can react with other reactive sulfur species to form mixed disulfides (–RSSR–) with protein thiols, *S*-glutathione adducts (–RSSG) with glutathione, or persulfides (–RSSH) with hydrogen sulfide (Willems *et al.*, 2021). The oxidation of protein Cys groups can be catalysed by enzymes, such as protein disulfide isomerases that introduce disulfide bridges during protein folding, or indirectly by thiol peroxidases via disulfide exchange reactions (Delaunay *et al.*, 2002; Veal *et al.*, 2002). These oxidation reactions are, however, reversible. The reduction reaction is catalysed by 'redoxin' enzymes, such as TRXs and GRXs that transfer reducing equivalents from the PET chain as well as from NAPDH, GSH, and ascorbate. Together, the ascorbate–glutathione cycle, and the redoxin systems, not only regulate but also protect protein thiols from overoxidation to sulfinic (–RSO2H) and sulfonic (–RSO3H) acids. The latter oxidation reactions are essentially irreversible and hence lead to protein inactivation. Moreover, these redox reactions form the basis for the post-translational modulation (PTM) of a wide range of proteins that regulate not only metabolism but also ROS signalling and protein–protein interactions, such as those of the chloroplast CP12-2/phosphoribulokinase (PRK)/glyceraldehyde 3-phosphate dehydrogenase (GAPB) ternary complex.

As mentioned above, the WWC is linked to OPDA signalling and GSH synthesis (Park *et al.*, 2013). Moreover, the activity of γ-glutamylcysteine synthetase (γ-ECS), which catalyses the first step of the committed GSH synthesis pathway, is regulated by oxidation, both at the level of oxidant-induced de-repression of γ-ECS translation and at the post-translational level by oxidation of enzyme thiol groups (Hicks *et al.*, 2007; Noctor *et al.*, 2012). The links between the WWC, GSH, and OPDA signalling are examples of the extensive crosstalk between the redox processing systems and hormone pathways that regulate plant defence systems. Similarly, redox changes associated with the ascorbate–glutathione cycle regulate retrograde signalling from chloroplasts and mitochondria to the nucleus in order to regulate gene expression that modifies plant growth and defence responses (Mielecki *et al.*, 2020).

Enzyme localization, properties, and functions

The enzymes of the ascorbate–glutathione cycle are localized in different intracellular compartments (Table 1). Very low levels of these enzymes have also been detected in the extracellular cell wall/apoplastic space (Vanacker *et al.*, 1998). APX1 has also been localized in the nuclei, together with SOD and CAT (Liu *et al.*, 2019; Foyer *et al.*, 2020a). Recent evidence suggests that the compartmentation of many enzymes associated with ROS processing or redox regulation is not as fixed as earlier concepts would suggest, and redox and other PTMs may facilitate re-localization of proteins to fulfil moonlighting functions (Foyer *et al.*, 2020b). The following discussion considers the enzymes of the ascorbate–glutathione cycle within this context.

The APXs are haem-containing enzymes that belong to class I of the peroxidase–catalase superfamily (Lazarotto *et al.*, 2021). APXs are encoded by small gene families, with different isoforms targeted to the cytosol, plastids, mitochondria, and peroxisomes (Lazarotto *et al.*, 2021). Some APX forms are associated with membranes, such as the plasmalemma, the peroxisomal membranes, and the thylakoid membranes, often together with MDHARs, while other APX forms are in the soluble phase. The APX forms differ in substrate affinities, dimer formation, and the presence of transmembrane domains. Moreover, the cytosolic APX of *Oncidium* orchid (*Og*cytAPX1) uses GSH as a substrate as well as ascorbate, but with different active sites (Chin *et al.*, 2019). The Pro63, Asp75, and Tyr97 residues are required for GSH oxidation by *Og*cytAPX1, whereas the corresponding site in *At*APX1 is composed of Asp63, His75, and His97, and has no GSH binding activity. In addition to *Og*cytAPX1, the recombinant cytosolic APX forms from maize, rice, and soybean also possess GSH oxidation activity (Chin *et al.*, 2019). Such interactions, like those linking GSH to the reduction of 2-Cys PRX, demonstrate that there are multiple additional levels of complexity to the ascorbate–glutathione cycle.

Some APX forms, such as Arabidopsis *At*APX1 (Kaur *et al.*, 2021) and the rice *Os*APX2 (Hong *et al.*, 2018), have chaperone functions. However, only the high molecular weight (HMW) complexes of *At*APX1 and *Os*APX2 display chaperone activity, whereas the LMW forms exhibit predominantly PRX activity (Hong *et al.*, 2018). These APX forms undergo structural and functional transitions between HMW and LMW forms. In addition, certain APX isoforms are highly sensitive to oxidative inactivation (Shikanai *et al.*, 1998). Hence, 2-Cys PRXs and other PRXs are required to ensure H_2O_2 processing in organelles, such as chloroplasts that produce large amounts of this oxidant.

Like other enzymes of the ascorbate–glutathione cycle, APXs are also subjected to PTMs. For example, the peroxidase activity of APX1 is regulated by *S*-nitrosation, tyrosine nitration, and *S*-sulfhydration either negatively or positively,

Table 1. The subcellular localization of the enzymes of the ascorbate–glutathione cycle

Enzyme	Isoforms	Localization	Species	Reference
APX	APX1, APX2, (APX6)	Cytosol	Arabidopsis, Sugarcane	Kaur *et al.* (2021)
	Stromal sAPX, Thylakoid tAPX	Chloroplast	Arabidopsis	Liu *et al.* (2018)
	APX3, (APX4)	Microsomes	Arabidopsis	Maruta *et al.* (2010)
				Jardim-Messeder *et al.* (2022)
				Narendra *et al.* (2006)
MDHAR	MDHAR1, MDHAR4	Peroxisomes	Arabidopsis, cotton	Lisenbee *et al.* (2005)
	MDHAR2, MDHR3	Cytosol		Zhou *et al.* (2021)
	MDHAR5	Mitochondria		
	MDHR6	Chloroplast		
DHAR	DHAR1	Peroxisomes? Cytosol?	Arabidopsis	Terai *et al.* (2020)
	DHAR2, DHAR3	Cytosol Chloroplast		Rahantaniaina *et al.* (2017b)
GR	GR1	Cytoplasm Nucleus Peroxisomes	Arabidopsis	Li *et al.* (2022)
	GR2	Mitochondria Plastids		Delorme-Hinoux *et al.* (2016)
				Amr *et al.* (2010)
				Marty *et al.* (2019)

depending on the plant species (Begara-Morales *et al.*, 2016; Aroca *et al.*, 2018). The Arabidopsis APX1 protein has five Cys residues, of which two (Cys32 and Cys49) are *S*-nitrosated (Yang *et al.*, 2015). The Cys32 residue is also the target for *S*-sulfhydration (Aroca *et al.*, 2015), which could regulate the binding affinity of APX1 for ascorbate, resulting in increased PRX activity. Tyrosine nitration has been also suggested to inhibit APX1 activity in pea and tobacco (Clark *et al.*, 2000; Begara-Morales *et al.*, 2013). Moreover, protein phosphorylation catalysed by the calcium-dependent protein kinase, CPK 28, activates APX2 activity through phosphorylation at Thr59 and Thr164 (Hu *et al.*, 2021). In contrast, a wheat kinase, called start 1.1, translocates to chloroplasts where it binds and phosphorylates tAPX, decreasing its activity and ability to remove H_2O_2 (Gou *et al.*, 2015). Crotonylation of protein Lys residues is an important PTM that has been recently shown to regulate many plant processes (Contreras-de la Rosa *et al.*, 2022). For example, crotonylation of Lys136 in the chrysanthemum APX increases enzyme activity to increase protection against low-temperature stress (Lin *et al.*, 2021).

Nitric oxide (NO) is an important regulator of ROS accumulation in plants through the regulated enhancement of the activities of ROS-scavenging enzymes, such as APX, CAT, and SOD, for example during stress responses (Beligni *et al.*, 2002; Xue *et al.*, 2007; Keyster *et al.*, 2011; Begara-Morales *et al.*, 2014). In the presence of molecular oxygen, NO undergoes an *S*-nitrosation reaction with GSH, forming GSNO, which leads to PTMs and nitration of proteins, such as APX (Correa-Aragunde *et al.*, 2015). While NO inhibits the activity of the cytosolic APX in tobacco Bright Yellow-2 suspension cells through *S*-nitrosation (de Pinto *et al.*, 2013), *S*-nitrosation positively regulates the activity of the Arabidopsis cytosolic APX1, upon exposure to stress (Yang *et al.*, 2015), and contributes to the suppression of cell death responses (Lin *et al.*, 2011). NO also regulates H_2O_2 levels and hence the shelf life and nutritional quality of pepper fruits through modulation of the different APX isozymes (González-Gordo *et al.*, 2022). NO reacts with O_2^- to produce peroxynitrite ($ONOO^-$), a molecule that can nitrate lipids, nucleic acids, aromatic rings, and the tyrosine residues in proteins leading to tyrosine nitration. This selective PTM can regulate enzyme activity, as well as preventing or promoting tyrosine phosphorylation.

MDHARs are typical FAD monomeric enzymes that catalyse redox reactions using FADH as substrate to reduce MDHA to ascorbate (Zhou *et al.*, 2021). MDHAR activity is crucial for enhancing the efficiency of the APX reaction in cellular compartments where the activities of these enzymes are coupled. MDHARs have been divided into three classes: class I, chloroplastic/mitochondrial enzymes; class II, peroxisomal membrane-attached enzymes; and class III, cytosolic/peroxisomal enzymes (Tanaka *et al.*, 2021). All plants have class II and III enzymes, which are the peroxisomal membrane-attached and cytosolic/peroxisomal isoforms, while some plants lack class I chloroplastic/mitochondrial enzymes. The chloroplast MDHAR forms are activated by TRXs. For example, the plastidial MDHAR form is activated by TRXy2, and the activity of a recombinant plastid Arabidopsis MDHAR isoform (MDHAR6) increases in the presence of reduced TRXy, and not other plastidial TRXs (Vanacker *et al.*, 2018).

In addition to MDHA, MDHARs can also use organic radicals as substrates (Hossain *et al.*, 1984). MDHARs recycle the oxidation products of other powerful antioxidants, such as phenolic compounds: ferulic acid, quercetin, chlorogenic acid, and coniferyl alcohol (Sakihama *et al.*, 2000). MDHAR6 reacts, for example, with the explosive 2,4,6-trinitrotoluene (TNT), generating superoxide (Johnston *et al.*, 2015). Plasma membrane

electron transport from ascorbate to MDHA has also been proposed, in a process that involves a high-potential plant plasma membrane cytochrome *b* (Horemans *et al.*, 1994). Moreover, NO scavenging by barley haemoglobin is facilitated by the MDHAR-mediated ascorbate reduction of methaemoglobin (Igamberdiev *et al.*, 2006).

The overexpression of *MDHAR* genes has consistently been shown to increase ascorbate accumulation and increase plant stress tolerance (Eltayeb *et al.*, 2007; Kavitha *et al.*, 2010; Li *et al.*, 2010; Yin *et al.*, 2010; Eltelib *et al.*, 2012; Yeh *et al.*, 2019). In contrast, mutants lacking MDHAR do not always show changes in ascorbate accumulation. For example, the peroxisomal membrane-associated ascorbate-dependent electron transfer system involves APX as well as MDHAR. While the Arabidopsis peroxisomal membrane APX isoform (APX3) is dispensable for growth and development (Narendra *et al.*, 2006), the seedling-lethal *sugar-dependent2* mutant is deficient in the peroxisomal membrane MDHAR isoform (MDHAR4). MDHAR4 mutants also have lower ascorbate to DHA ratios, but have similar total ascorbate levels to the wild type (Eastmond, 2007). Taken together, these findings suggest that other system enzymes, in addition to MDHAR, may not be a rate-limiting step in ascorbate recycling.

The DHAR enzymes belong to the glutathione *S*-transferase (GST) superfamily and have a characteristic two-domain architecture, comprised of a mixed α/β N-terminal domain containing the glutaredoxin motif (CXX[C/S]) and an all-helical C-terminal domain (Littler *et al.*, 2010). The active site comprises a glutathione-binding G-site and a hydrophobic substrate-binding H-site. The reaction probably proceeds via a 'ping–pong' mechanism, where DHA binds to the free reduced form of DHAR followed by binding of GSH (Ding *et al.*, 2020).

The requirement and functions of the DHARs in ascorbate regeneration have, however, long been a matter of debate (Morell *et al.*, 1997, 1998; Foyer and Mullineaux, 1998). Genetic studies using DHAR overexpression, knockdown, and/or knockout lines supported the physiological importance of DHARs in ascorbate recycling (Chen *et al.*, 2003; Chen and Gallie, 2004, 2006, 2008; Gallie, 2013; Noshi *et al.*, 2016, 2017). For example, the multivitamin white corn variety with high DHAR activity has a 6-fold higher kernel ascorbate level than controls (Naqvi *et al.*, 2009). Moreover, DHAR gene expression is also associated with enhanced abiotic stress tolerance (Broad *et al.*, 2020). Loss-of-function mutations in the Arabidopsis cytosol-targeted DHAR2 form alone led to lower ascorbate/DHA ratios but did not affect total ascorbate accumulation (Yoshida *et al.*, 2006). Nevertheless, the physiological role of DHARs remains uncertain, largely because the Arabidopsis triple-knockout (*dhar1 dhar2 dhar3*) mutants that lack all three DHARs have negligible DHAR activity and display similar levels of ascorbate to the wild-type controls, with ascorbate/DHA ratios as well as plant growth and development similar to the wild type (Rahantaniaina *et al.*, 2017a, b). In addition, the absence of DHAR activity had no impact on the ascorbate profiles of the catalase-deficient mutant (*cat2*) that maintains a highly oxidized glutathione pool. DHAR activity was also required for the GGSG accumulation and cell death phenotypes that are observed in the *cat2* mutants under stress conditions (Rahantaniaina *et al.*, 2017a). Moreover, DHAR activity was required to maintain ascorbate recycling capacity under high light conditions in the *phytoalexin-deficient 2-1* (*pad2-1*) mutants that have low glutathione accumulation (Terai *et al.*, 2020). Hence, multiple systems including MDHAR, DHAR, glutathione, and ferredoxin contribute to the generation of reduced ascorbate. For example, the CPYC-type GRXs exhibit DHAR activity (Sha *et al.*, 1997; Rouhier *et al.*, 2003). Other as yet uncharacterized proteins may also have DHAR activity (Morell *et al.*, 1997). Nevertheless, current evidence suggests that GSH is required for ascorbate regeneration under high light conditions (Terai *et al.*, 2020). DHAR activity also maintained the ascorbate pool in mutants that have low ascorbate accumulation (Terai *et al.*, 2020), and other recycling systems contribute to ascorbate recycling when ascorbate levels are high.

GRs are responsible for maintaining the cellular glutathione pools in the reduced state. As such, these flavoprotein oxidoreductases are crucial regulators of plant development and the responses to environmental stress (Foyer *et al.*, 1995; Noctor *et al.*, 1998). GSH is a central signalling molecule in plants that functions together with the GRX and TRX systems to regulate numerous phytohormone-associated pathways (Rai *et al.*, 2023). It also serves as a cofactor for various enzymes, such as GRXs and GSTs, which play crucial roles in cell detoxification pathways. A recent study also proposed that GSH, together with neodiosmin, is a signature metabolite for pattern-triggered immunity and effector-triggered immunity involving surface-localized pattern recognition receptors and intracellular nucleotide-binding leucine-rich repeat receptors (Lu *et al.*, 2023).

Higher plant GRs are encoded by two genes: *GR1* and *GR2*. While *GR1* encodes a cytosolic, or peroxisomal, form of the enzyme, *GR2*, which contains a long N-terminal sequence, encodes a mitochondrial and chloroplastic form. The chloroplast form represents ~80% of the total GR activity. GR has also two Cys residues that form a redox-active disulfide bridge at the active site. Glutathione disufide binds to the active site to form a disulfide bond separately with a Cys residue and a His residue at the active site allowing reduction to GSH (Kataya and Reumann, 2010; Yousuf *et al.*, 2012; Couto *et al.*, 2016). Overexpression of the chloroplast form of GR significantly increases the GSH content of plants and increases tolerance to a range of abiotic stresses (Foyer *et al.*, 1995; Li *et al.*, 2005; Gill *et al.*, 2013). The chloroplast-localized GR2 also fulfils essential roles in root apical meristem maintenance (Yu *et al.*, 2013).

GRXs are thioltransferases that serve a number of important roles in plants (Meyer *et al.*, 2008, 2012, 2021). These small (12 kDa) redox enzymes catalyse not only the reduction

of disulfides, but also the reduction of mixed disulfides, in a process called deglutathionylation. Hence, they act as oxidoreductases that control glutathionylation/deglutathionylation reactions. GRX functions depend on two distinct interaction sites for efficient redox catalysis (Begas *et al.*, 2017). The first site interacts with the GSH moiety of glutathionylated disulfide substrates. The second site activates GSH as the reducing agent (Begas *et al.*, 2017). There are five GRX subgroups that are classified according to their active site sequences, of which groups III and IV are specific to vascular plants. In *Arabidopsis thaliana*, group I proteins that have C[P/G/S]Y[C/S] in the catalytic site are localized in the cytosol and plastids and are encoded by six genes. The group I GRXs undertake oxidoreductase functions and are found in most organisms. The four group II (monothiol GRXs or CGFS GRXs) GRXs in *A. thaliana* are localized in the cytosol, plastid, nucleus, and mitochondria. The third type of GRXs have a Cys-Cys-X-Cys or Cys-Cys-X-Ser sequence at the active site and are specific to higher plants. They are also called ROXY GRXs (Zaffagnini *et al.*, 2008; Ströher and Millar, 2012). There are 21 members of group III in *A. thaliana* that are localized in the cytoplasm and nucleus. Group IV proteins have a GRX domain followed by four CxxC repeats at the C-terminus (Navrot *et al.*, 2006). Group V (CPF[C/S]) has six members that are found in the cytosol, mitochondria, and chloroplast.

Class II GRXs act as iron–sulfur (Fe–S) cluster bridging proteins. They function as maturation factors for the production of Fe–S proteins (Rey *et al.*, 2019). As such, they participate in iron homeostasis and the maturation of Fe–S protein [2Fe–2S] clusters with interacting proteins. For example, the GRX [2Fe–2S] clusters form complexes with BOLA proteins, in which the [2Fe–2S] cluster is ligated using the GRX conserved Cys, a Cys from GSH, and His or Cys residues on the BOLA protein. The function of the plastid GRXs as Fe–S cluster bridging proteins links them to the thylakoid membrane functions and chlorophyll metabolism. Like PRXs and TRXs, the GRX proteins may serve functions in organelle to nucleus retrograde signalling pathways (Sevilla *et al.*, 2023).

Moonlighting functions

Many of the proteins involved in the ascorbate–glutathione cycle reside in different intracellular compartments where they can have 'moonlighting' as well as enzymatic functions. APXs have a broad substrate specificity and possess chaperone activity, hence participating in various biological processes (Li, 2023). Of the eight *AtAPX* genes in *A. thaliana*, three encode cytosolic (cytAPXs: *At*APX1, 2, and 6) proteins, three microsomal/peroxisomal (perAPXs: *At*APX3, 4, and 5) proteins, and two chloroplastic (chlAPXs: soluble stromal *At*sAPX and thylakoid membrane-bound *At*tAPX) protein isoforms (Panchuk *et al.*, 2005; Granlund *et al.*, 2009). Like the rice *Os*APX2 protein (Hong *et al.*, 2018), *At*APX1 has chaperone functions (Kaur *et al.*, 2021). The LMW forms of *At*APX1 and *Os*APX2 exhibit peroxidase activity, but the HMW complexes also display chaperone activity.

The *At*APX4 and *At*APX6 (APX-L and APX-R) proteins lack essential catalytic residues, ASC-binding sites, and haem-binding sites (Granlund *et al.*, 2009). These proteins, which are generally encoded by a single gene, have been reclassified as two novel families of class I peroxidases (Lazzarotto *et al.*, 2015). The chloroplast-targeted *At*APX6 protein is also found in the cytosol and functions as a haem peroxidase that does not use ascorbate as a substrate to reduce H_2O_2 (Lazzarotto *et al.*, 2021). APXs can also oxidize non-physiological aromatic substrates *in vitro*, such as *p*-cresol, *o*-dianisidine, and guaiacol, at rates comparable with ascorbate (Raven, 2003). For example, the soluble cytosolic coumarate 3-hydroxylase (C3H) enzymes of *A. thaliana* and *Brachypodium distachyon* can oxidize both ascorbate and 4-coumarate at comparable rates (Barros *et al.*, 2019).

No moonlighting functions have as yet been reported for MDHAR proteins, which can reduce a wide range of substrates in addition to DHA. However, the class II enzymes attach to the peroxisomal membrane and have essential roles in plant development (Eastmond, 2007). The *A. thaliana At*MDAR4 protein, which binds to the peroxisomal membrane, protects the SDP1 triacylglycerol lipase from oxidation, but the mechanistic reasons for this phenotype are unknown. The *sdp2* mutants that lack the class II *At*MDHAR4 enzyme have a seedling-lethal phenotype in the absence of exogenous sugar treatment. The siliques of *mdar1-2*$^{(+/-)}$ *mdar4-5*$^{(-/-)}$ double mutants have both normal and empty seeds, whereas those of the wild type and single mutants have only normal seeds, suggesting that the double knockout of both isoforms causes embryonic lethality (Tanaka *et al.*, 2021).

Plant DHARs are dimorphic proteins that exist in soluble enzymatic and membrane-integrated forms. They share a structural glutathione *S*-transferase (GST) fold with human chloride intracellular channels (HsCLICs). HsCLICs are dimorphic proteins that exist in soluble enzymatic and membrane-integrated ion channel forms. *At*DHAR1 is able to generate inward conductance in transfected mammalian cell membranes (Das *et al.*, 2016) and the *Pennisetum glaucum* (*Pg*)DHAR is dimorphic and has been localized in the plasma membrane (Das *et al.*, 2023). Thus, DHAR can function as an oxidative stress-regulated ion channel (Das *et al.*, 2023).

Support for ascorbate functions in plants

Ascorbate is a multifunctional metabolite (Table 2) that regulates plant growth and development (Foyer *et al.*, 2020b). It is a major non-enzymatic antioxidant and ubiquitous ROS scavenger that is better (>100× faster) than GSH at scavenging superoxide and singlet oxygen. As such, it interacts with various redox regulatory signalling networks and plays a key role

Table 2. The functions of ascorbate in plants

Function	Target	Reference
ROS processing	Removal of superoxide and hydrogen peroxide (e.g. produced in photosynthesis; Arabidopsis)	Awad *et al.* (2015); Karpinska *et al.* (2017); Foyer *et al.* (2020)
Antioxidant regeneration	α-Tocopherol reduction	Munne-Bosch (2005)
Electron donor/acceptor	(PSI/PSII) (e.g. barley, Arabidopsis)	Mano *et al.* (2004); Tóth *et al.* (2009)
Enzyme cofactor	Peroxidase substrate (e.g. ascorbate peroxidase; poplar)	Miyake and Asada (1994); Mehlhorn *et al.* (1996)
	De-epoxidation (violaxanthin de-epoxidase; rice)	Müller-Moulé *et al.* (2002); Saga *et al.* (2010)
	Hydroxylation (Fe- and 2-oxoglutarate-dependent dioxygenases; ethylene, GA, ABA) (e.g. tomato, rice, Arabidopsis)	Wei *et al.* (2021); Smirnoff (2018); Bulley *et al.* (2021); Ye and Zhang (2012)
	Flavonoid biosynthesis (Arabidopsis)	Page *et al.* (2012)
Enzyme inhibitor	Chloroplast antioxidant enzyme (Arabidopsis)	Horling *et al.* (2003)
Flower development	Anther/pollen development (e.g. orchid, Arabidopsis)	Deslous *et al.* (2021)

in redox signal transduction, particularly in relation to abiotic stress tolerance. For example, ascorbate was found to have a specific and light-dependent effect on the expression of the gene encoding the chloroplast 2-Cys peroxiredoxin-A2, an effect that could not be substituted by GSH (Shaikhali and Baier, 2016). The concentration of ascorbate in *A. thaliana* cells is the lowest in the vacuoles (2.3 mM), with higher levels in the mitochondria (10.4 mM), chloroplasts (10.8 mM), and nuclei (16.3 mM). The highest ascorbate concentrations were found in the cytosol (21.7 mM) and peroxisomes (22.8 mM) (Zechmann, 2011; Zechmann *et al.*, 2011). In comparison, the concentrations of ascorbate (0.002 mM) and DHA (0.36 mM) in the apoplast are relatively low (Booker *et al.*, 2012).

Ascorbic acid can be as efficient as SOD in catalysing the removal of superoxide radical (Som *et al.*, 1983). The rate constant for the reaction between ascorbic acid and superoxide (at pH 7.4) using the xanthine–xanthine oxidase system was estimated to be 5.4×10^6 M^{-1} s^{-1} (Som *et al.*, 1983). However, Gray and Carmichael (1992) reported that the rate constant for bovine erythrocyte Cu,Zn-SOD was kSOD=6.4×10^9 M^{-1} s^{-1} which is 1000 times higher. Nevertheless, the lifetime of superoxide as a signalling molecule can be considered to depend on the presence of SODs and ascorbate, which essentially police this molecule. Superoxide accumulation in plant stem cells such as those found in the shoot apical meristem (SAM) and the root apical meristem (RAM) is important in defining the identity of undifferentiated meristematic cells (Tsukagoshi *et al.*, 2010; Zeng *et al.*, 2017). Like SODs, ascorbate is largely absent from the quiescent centre of the RAM. The addition of ascorbic acid stimulates not only the activity of the quiescent centre cells but also cell proliferation in the entire root meristem (Liso *et al.*, 1998).

Ascorbate may also play a key role in policing organelle to nucleus communication and signalling pathways. For example, mutations in proteins such as the rice chloroplast-localized pseudouridine synthase (OSPUS 1-1) lead to the production of albino seedlings under low temperatures because of aberrant chloroplast ribosome biogenesis (Wang *et al.*, 2022). Overexpression of mitochondrial MnSOD also rescues the phenotype, as does the suppressor protein of *ospus 1-1*, which encodes a mitochondrial pentapeptide repeat (PPR) protein. Such findings suggest that there is coordinated superoxide signalling between the mitochondria and chloroplasts that regulates plastid development. The chloroplast ascorbate–glutathione system, particularly the chloroplast APXs, has been found to regulate signalling related to stress experiences, such as low temperature stress, over time without the requirement of establishing cold acclimation (van Buer *et al.*, 2016). Moreover, cold priming was found to modify chloroplast to nucleus signalling by thylakoid APX-mediated suppression of CEF mediated by the thylakoid NADH dehydrogenase complex (Seiml-Buchinger *et al.*, 2022).

Dry seeds are devoid of reduced ascorbate and APX activity. They contain only DHA, suggesting that the ascorbate–glutathione cycle does not function in dry seeds. Clearly the reduced ascorbate content of plant organs has to be maintained within certain thresholds, according to tissue requirements. Attempts to enhance ascorbate levels must therefore be approached with caution because artificially high ascorbate levels as a consequence of removing feedback controls were shown to impair reproductive development (Deslous *et al.*, 2021).

Ascorbate is also an essential enzyme cofactor that participates in the regulation of photosynthesis and metabolism. It is a specific cofactor for a large family of enzymes known as the Fe- and 2-oxoglutarate-dependent dioxygenases that catalyse the addition of a hydroxyl group to various substrates (Wei *et al.*, 2021). Ascorbate is required for the maintenance of activity of Fe(II)/2-oxoglutarate-dependent dioxygenases via reduction of Fe(III). As such, ascorbate is involved in the synthesis of phytohormones and secondary metabolites. For example, ascorbate is required for opening the ring structure of 1-aminocyclopropane-1-carboxylic acid (ACC) by supplying the electron to the active site of ACC oxidase, which catalyses the last step of ethylene biosynthesis (Smirnoff, 2018). Ascorbate has also been implicated in auxin catabolism and the synthesis of abscisic acid and gibberellins through its functions with different dioxygenases.

Ascorbate is the natural substrate for many types of plant peroxidases (Mehlhorn *et al.*, 1996). In this way, ascorbate influences the accumulation of a wide range of phenolic compounds, particularly in the cell wall/apoplastic compartment of plant cells. Ascorbate regulates the expression of genes involved in flavonol and anthocyanin precursor synthesis (Page *et al.*, 2012) such as PHENYLALANINE AMMONIA-LYASE1 (PAL1), 4-COUMARATE:COENZYME A LIGASE3, CHALCONE SYNTHASE (CHS), as well as the MYB transcription factor PAP1 and an ELONGATED HYPOCOTYL5 (HY5) homologue HYH (Munné-Bosch *et al.*, 2013). The low levels of leaf ascorbate in ascorbate-deficient mutants (*vtc2-1* and *vtc2-4*) causes, however, a significant decrease in leaf anthocyanin contents (Plumb *et al.*, 2018).

Leaf ascorbate accumulation is modulated by the amount and quality of light. Leaf ascorbate accumulation is lowest at night and highest at the end of the day. Similarly, increases in the light red/far red ratios (a 'shade' phenotype) resulted in much lower leaf ascorbate and GSH contents than high red/far red ratios (Bartoli *et al.*, 2009; Foyer *et al.*, 2020b). Blue light has been shown to activate the expression of the gene encoding GDP-L-galactose phosphorylase (GGP), which is the main controlling step of the L-galactose pathway of ascorbate synthesis (Bournonville *et al.*, 2023). This protein resides in the cytoplasm and the nucleus, where it interacts with the PAS/LOV photoreceptor protein (PLP) to mediate light-dependent control of ascorbate synthesis. PLP is a non-competitive inhibitor of GGP that is inactivated upon exposure to blue light (Bournonville *et al.*, 2023). Light increases APX, MDHAR, and GR activities. Light-dependent regulation of APX and MDHAR activities of these enzymes occurs via PTMs as well as at the level of gene expression (Gulyás *et al.*, 2023).

The Arabidopsis *vtc2/vtc5* double mutants, which are unable to synthesize ascorbate, are not viable (Dowdle *et al.*, 2007). Mutants that have a low ascorbate content have significant reprogramming of gene expression, including genes involved in hormone synthesis and signalling, as well as photosynthesis and defence (Kiddle *et al.*, 2003; Pastori *et al.*, 2003). These changes are accompanied by increases in the levels of salicylic acid (SA), pathogenesis-related proteins, and camalexin that demonstrate the activation of the ROS signalling branch of plant innate immunity (Pavet *et al.*, 2005; Mukherjee *et al.*, 2010). In this way, ascorbate can exert a key role in plant immunity, as well as defence responses to abiotic environmental stresses (Pastori *et al.*, 2003; Pavet *et al.*, 2005; Venkatesh and Park, 2014; Akram *et al.*, 2017) including salt stress (Shalata *et al.*, 2001). Ascorbate accumulation is also important in the regulation of plant defences against biotrophic pathogens that rely on SA signalling such as *Pseudomonas syringae* and *Peronospora parasitica* (Pavet *et al.*, 2005; Mukherjee *et al.*, 2010) as well as phloem-feeding insects (Kerchev *et al.*, 2013). In contrast, ascorbate deficiency enhances susceptibility to the necrotrophic pathogen *Alternaria brassicicola*, in which defence is mediated by jasmonic acid and ethylene signalling (Botanga *et al.*, 2012). The application of exogenous ascorbate also acts as an inducer of disease resistance in plant interactions with different types of pathogens including viruses (Fujiwara *et al.*, 2013). The mechanisms involved in such strategies are complex, because reduced ascorbate is highly susceptible to oxidation in aqueous solution and, moreover, it is likely to be oxidized by the ascorbate oxidase activities in the apoplast/cell wall compartments of the plant cell before it enters the cytoplasm. The role of ascorbate in programmed cell death (PCD) is related to its role in the control of the activation of the ROS signalling branch of innate immune responses (Pavet *et al.*, 2005; Mukherjee *et al.*, 2010) Localized PCD, similar to that occurring during hypersensitive responses to plant pathogens, is observed in the leaves of ascorbate-deficient mutants (Pavet *et al.*, 2005). Increased ascorbate synthesis, resulting from supplying L-galactono-1,4-lactone, delays PCD during kernel maturation in durum wheat, with a consequent postponement of dehydration and improvement in kernel filling (Paradiso *et al.*, 2012).

Ascorbate may also influence plant epigenetic processes (Ramakrishnan *et al.*, 2022; Seiml-Buchinger *et al.*, 2022). Ascorbate is a cofactor for the ten−eleven translocation (TET1−TET3) family of proteins in mammalian cells, which are responsible for the removal of cytosine methylation in DNA (Zhithovich, 2020). Ascorbate drives the active removal of this transcription-repressive mark by enhancing the activities of TET enzymes. The TET enzymes are Fe(II)-dependent dioxygenases that catalyse a series of consecutive oxidations of 5-methylcytosine. No TET-like enzymes have as yet been identified in plants, although 5-methylcytosine oxidation products, particularly 5-hydroxymethylcytosine (5hmC), have been found in plants (Mahmood and Dunwell, 2019). However, superoxide may influence the activities of proteins that contain the [Fe–S] clusters that mediate the regulation of DNA demethylation in a manner that is regulated by ascorbate.

Ascorbate fulfils a number of important roles in the regulation of photosynthesis, particularly in the acclimation of plants to high light (Müller-Moulé *et al.*, 2014; Karpinska *et al.*, 2017). In addition to its participation in the WWC, ascorbate is also required for the regeneration of lipid-soluble antioxidants, particularly tocopherols and tocotrienols (vitamin E), which protect the polyunsaturated fatty acids in the thylakoid membranes from oxidation to chromanoxyl radicals by singlet oxygen. These radicals are converted back to vitamin E by the reducing power of ascorbate, or by reaction with carotenoids. Ascorbate is also required for the conversion of violaxanthin to zeaxanthin in the light-dependent xanthophyll cycle, which is a key component of the thermal energy dissipation mechanisms measured by the non-photoenergy quenching component of Chl *a* fluorescence (Müller-Moulé *et al.*, 2002). Knockout mutants of the chloroplast envelope ascorbate transporter *At*PHT4;4 are compromised in thermal energy dissipation (Miyaji *et al.*, 2015). Moreover, ascorbate is a potent specific inhibitor of the expression of 2-Cys PRX A and other

chloroplast antioxidant enzymes (Horling *et al.*, 2003; Baier *et al.*, 2004). This influences chloroplast to nucleus signalling pathways via the redox-sensitive transcription factor Rap2.4a (Shaikhali *et al.*, 2008). Conversely, the expression of chloroplast APX and MDHAR is induced in lines defective in 2-Cys PRXs (Baier *et al.*, 2000). Ascorbate is finally also able to donate, as well as accept, electrons from the PET chain, acting as an alternative electron donor for PSII (Mano *et al.*, 2004; Tóth *et al.*, 2009).

While ascorbate has been largely discounted as a significant factor in NO metabolism (Wang and Hargrove, 2013), the ascorbate-mediated regulation of flowering in plants, such as in *Oncidium*, acts through the NO-mediated flowering-repression pathway (Kumar *et al.*, 2016). Arabidopsis low ascorbate mutants have long been known to show early flowering (Barth *et al.*, 2006), a trait that is linked to the altered expression of genes, such as flowering locus T (*FT*) and CONSTANS (*CO*) that regulate flowering (Kotchoni *et al.*, 2009). Similar effects on flowering have been reported for plants with altered APX or ascorbate oxidase (AO) activities (Pnueli *et al.*, 2003; Pignocchi *et al.*, 2006). Moreover, the exogenous application of ascorbate or its precursor L-galactono-1,4-lactone delays flowering (Shen *et al.*, 2009).

Support for glutathione functions

Reduced glutathione (γ-glutamyl-cysteinyl-glycine: GSH) is one of the most abundant LMW non-protein thiols in plants. GSH reacts with superoxide and H_2O_2, but this reaction is relatively slow compared with ascorbate (Winterbourn, 2016). Nevertheless, GSH is an essential metabolite with a wide range of important functions in plant biology (Noctor *et al.*, 2012; Hasanuzzaman *et al.*, 2017; Aslam *et al.*, 2021; Dorion *et al.*, 2021; Dumanović *et al.*, 2021). The glutathione redox couple (GSH/GSSG) functions together with other redox-active couples, such as NADPH/$NADP^+$ and TRX-SH/TRX-SS, to maintain cellular redox homeostasis and propagate redox signals (Foyer and Noctor, 2011; Considine and Foyer, 2021; Le Gal *et al.*, 2021).

GR activity ensures that plant cells maintain very high GSH:GSSG ratios. Decreases in GSH:GSSG ratios stimulate the reversible formation of mixed disulfides between protein sulfhydryl groups and GSSG (i.e. *S*-glutathionylation), as well as GSH synthesis. *S*-Glutathionylation of proteins results in structural and functional modifications in redox-sensitive enzymes, that can, for example, regulate PET and plant immune responses (Grek *et al.*, 2013). The 2-Cys PRX proteins are glutathionylated by GSSG, a process that favours dimerization and inactivates their molecular chaperone activities (Park *et al.*, 2011). OPDA signalling also modulates GSH-dependent protein glutathionylation in a manner that regulates PET efficiency, as well as defence gene expression.

GPXs are, therefore, considered as part of the glutathione/ascorbate cycle. Plant GPX protein sequences have high sequence similarities to mammalian phospholipid hydroperoxide GPX4 (Faltin *et al.*, 2010), containing three conserved non-selenium Cys residues at the active sites. However, they catalyse the reduction of H_2O_2 using TRX and GRX as the electron donor rather than GSH. They are, therefore, more correctly called thiol peroxidases than GPXs (Bela *et al.*, 2015). The plant GPX protein family consists of multiple isoenzymes located in different subcellular compartments that have distinct expression patterns with respect to tissues and developmental stages (Gao *et al.*, 2014). These enzymes play an important role in protection against environmental stress (Zhang *et al.*, 2019). For example, transgenic plants overexpressing GPX genes have better stress tolerance (Diao *et al.*, 2014; Zhang *et al.*, 2019). Some GSTs also have GPX activity. These enzymes can detoxify lipid hydroperoxides and thus participate in antioxidative defence (Dixon *et al.*, 2005; Ding *et al.*, 2020). Plant GSTs are finally mostly cytosolic enzymes, and they can represent up to 2% of soluble proteins (Pascal and Scalla, 1999).

As discussed above, GRXs play important but non-overlapping roles in iron trafficking and the biogenesis of iron-containing cofactors (Berndt *et al.*, 2021). For example, GRX17 is required for the maturation of cytosolic and nuclear Fe–S proteins, with both foldase and a redox-dependent holdase functions in cluster biogenesis that are important for stress tolerance (Martins *et al.*, 2020). GRXs participate in the regulation of plant growth and development, as well responses to environmental triggers. For example, the class III GRXS3/4/5/8 proteins function downstream of cytokinins in Arabidopsis to negatively regulate primary root growth in response to nitrate (Patterson *et al.*, 2016). These GRXs mediate cytokinin-dependent responses, acting downstream of type-B response regulators that mediate the transcriptional responses to cytokinin to inhibit root growth in response to high nitrogen (Patterson *et al.*, 2016). In particular, AtGRXS8 represses the transcriptional and developmental responses of the primary root to nitrate, by interfering with the activity of the TGA1 and TGA4 transcription factors (Ehrary *et al.*, 2020).

GSH interacts with NO, forming *S*-nitrosoglutathione, which can sequester iron in LMW compounds named mono- and dinitrosyl iron complexes. GSNO functions as a mobile reservoir of NO, which is regulated in cells by the activity of GSNO reductases that modulate NO levels in plant cells (Sakamoto *et al.*, 2002; Corpas *et al*, 2013). Protein *S*-nitrosation is reversed by TRXs and *S*-nitrosoglutathione reductases (glutathione-dependent formaldehyde dehydrogenases). GSH works together with TRXs in a range of other processes, such as the redox control of PCD. A thiol-redox switch mechanism involving TRX and GSH mediates the propagation of apoptosis signals and acts as a redox checkpoint in mammalian cells (Benhar, 2020) In this system, the nitration of various proteins controls PCD in a manner that is reversed by TRX and GSH (Benhar, 2020).

Conclusions and perspectives

While a major function of the ascorbate–glutathione cycle is the policing of H_2O_2 signalling in the different subcellular compartments and also the intensity of the cell-to-cell ROS signalling wave, it also maintains the essential and multifaceted functions of ascorbate and GSH in plants. For example, ascorbate and GSH support the activities of different enzyme systems that fulfil important functions in plant growth and development. Moreover, ascorbate functions as a much more efficient superoxide scavenger than GSH and hences polices superoxide-dependent activities and signalling. The diverse functions of ascorbate and glutathione in plant biology require that the enzymes of the ascorbate–glutathione cycle do not always operate in synchrony. Clearly, the reduction of MDHA and DHA does not always require GSH, particularly in compartments in which the reduction of these metabolites by other systems is rapid, such as occurs in the vicinity of the PET chain. Similarly, the transport systems for the reduced and oxidized forms of ascorbate and glutathione facilitate the exchange of these metabolites between different compartments in a manner that remains poorly characterized. Thus, a number of factors including competing reactions, and the regulation of metabolite synthesis, degradation, and compartmentation determine whether GSH turnover is coupled to ascorbate turnover. The factors that integrate the pathways of ascorbate and GSH synthesis, recycling, and degradation remain poorly understood, although the compartmentation of these different processes is likely to be an important control point. While our understanding of the regulation of the enzymes of the pathway has greatly increased, some aspects such as the moonlighting functions remain to be fully elucidated.

While the functions of the ascorbate–glutathione cycle are well characterized in some organelles such as the chloroplasts and peroxisomes, virtually nothing is known about the roles of ascorbate and glutathione in the nucleus. Accumulating evidence suggests that superoxide and H_2O_2 are produced in the nucleus, where they fulfil important regulatory functions (Diaz-Vivancos *et al.*, 2015; de Simone *et al.*, 2017 García-Giménez *et al.*, 2017). For example, superoxide accumulation is required to maintain shoot meristem cells and the undifferentiated meristematic cells in the root (Zeng *et al.*, 2017; Zhao *et al.*, 2023, Preprint). Little is known about how the levels of superoxide are regulated to maintain cell fate within the stem cell niche, but modulation of SOD and the ascorbate–glutathione cycle are important in the control of this system. In particular, the roles of superoxide and SOD in plant nuclei are poorly documented. In breast cancer cells, acetylation converts SOD2 from a mitochondrial antioxidant to a nuclear histone demethylase to promote cell stemness and promotes cancer cell evolution (Coelho *et al.*, 2022). In this situation, FeSOD functions as a H3 histone demethylase that requires H_2O_2 as a substrate (Coelho *et al.*, 2022). The nuclei of plant cells are rapidly oxidized in response to stresses, such as high temperatures (Babbar *et al.*, 2021).

The metabolites and proteins that contribute to ROS production in the nucleus remain to be identified. However, the direct impacts of stress-induced oxidation of nuclei have significant implications for current concepts of redox sensing and regulation, as well as associated signal transduction pathways (Sevilla *et al.*, 2023). The control of nuclear thiol–disulfide redox states by nucleoredoxins and TRX1 remains, however, largely uncharacterized (Kneeshaw *et al.*, 2017). Similarly, how the nuclear glutathione and ascorbate pools influence the regulation of cell cycle proteins is also still not clear (Diaz-Vivancos *et al.*, 2015; de Simone *et al.*, 2017).

In conclusion, the ascorbate–glutathione cycle sits at the heart of redox biology and interacts on multiple levels with the wider network of oxidants, ROS processing proteins, and antioxidants that regulate every aspect of plant biology. There is now a huge literature on the ascorbate–glutathione cycle, including new and important findings that add context and complexity to cycle functions. The wider significance of the ascorbate–glutathione cycle is only now becoming apparent, as new signalling mechanisms, systems, and pathways are identified.

Author contributions

CF: conceptualization and preparing the manuscript text; KK: conceptualization, preparation of the figures and tables, and editing the text.

Conflict of interest

The authors have no conflicts of interest to declare

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Data availability

This manuscript does not contain original data

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