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Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept

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ABSTRACT

Plants suffering from abiotic stress are commonly facing an enhanced accumulation of reactive oxygen species (ROS) with damaging as well as signalling effects at organellar and cellular levels. The outcome of an environmental challenge highly depends on the delicate balance between ROS production and scavenging by both enzymatic and metabolic antioxidants. However, this traditional classification is in need of renewal and reform, as it is becoming increasingly clear that soluble sugars such as disaccharides, raffinose family oligosaccharides and fructans – next to their associated metabolic enzymes – are strongly related to stress-induced ROS accumulation in plants. Therefore, this review aims at extending the current concept of antioxidants functioning during abiotic stress, with special focus on the emanate role of sugars as true ROS scavengers. Examples are given based on their cellular location, as different organelles seem to exploit distinct mechanisms. Moreover, the vacuole comes into the picture as important player in the ROS signalling network of plants. Elucidating the interplay between the mechanisms controlling ROS signalling during abiotic stress will facilitate the development of strategies to enhance crop tolerance to stressful environmental conditions.

Key-words: antioxidants; oxidative stress; reactive oxygen species; vacuole.

INTRODUCTION

As sessile organisms in a continuously changing environment, plants are inevitably subjected to a diverse array of biotic and abiotic stressors. Pathogen attacks, soil salinity, drought, temperature changes, UV radiation, metals and herbicides such as paraquat negatively affect both crop yield and quality. A major reason for this is oxidative damage caused by an increased accumulation of reactive oxygen species (ROS; Suntres 2002; Frohnmeyer & Staiger 2003; Suzuki & Mittler 2006; Torres, Jones & Dangl 2006; Sharma & Dietz 2009; Bolouri-Moghaddam *et al*. 2010; Miller *et al*. 2010). Under optimal physiological conditions, ROS such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) are constantly produced as by-products of aerobic metabolism in chloroplasts, mitochondria and peroxisomes. Over the years, a dual role for ROS as both damaging and signalling compounds *in planta* has been established (Mittler *et al*. 2004; Møller & Sweetlove 2010). The enhanced ROS accumulation occurring in stressed plants is due to a disrupted balance between ROS production, avoidance and scavenging at any given cellular location and time (Mittler 2002; Mittler *et al*. 2004). Whereas increased ROS levels under stress are life-threatening by oxidising lipids, proteins and nucleic acids, they can also act as signals activating stress and defence pathways. The outcome of an environmental challenge therefore depends on the delicate balance between ROS producing and scavenging mechanisms, as well as stress intensity and duration (Miller *et al*. 2010).

In plants, stress-induced ROS accumulation is counteracted by two different processes: (1) prevention or avoidance of ROS formation and (2) actual ROS scavenging by both enzymatic and non-enzymatic low molecular metabolic antioxidants (Mittler 2002; Gill & Tuteja 2010). Next to well-known pro- and antioxidants, soluble sugars have a dual role with respect to ROS. They can either be involved in ROS producing metabolic pathways, but can also funnel NADPH-producing metabolism such as the oxidative pentose-phosphate (OPP) pathway and thereby contribute to ROS scavenging (Couée *et al*. 2006; Bolouri-Moghaddam *et al*. 2010). However, in the emerging 'sugar as antioxidant' concept, it is becoming increasingly clear that sugars, especially those interacting with membranes (Bolouri-Moghaddam *et al*. 2010), can also act as true ROS scavengers *in planta* (Van den Ende & Valluru 2009; Peshev & Van den Ende 2013). Moreover, similar mechanisms might counteract ROS-related diseases in the human body (Van den Ende, Peshev & De Gara 2011).

In this review, our current knowledge on ROS production, avoidance and scavenging will be discussed in the light of abiotic stress in plants, with special attention on the emanate role of sugars as antioxidants. Examples will be given based on their cellular location, as different mechanisms are exploited in different plant organelles. As abiotic stress is estimated to be the head cause of crop loss exceeding 50% worldwide (Cramer *et al*. 2011), it is highly important to elucidate the mechanisms controlling ROS signalling pathways and their interplay during abiotic stress. This can finally contribute to the development of strategies enhancing crop tolerance to environmental stress conditions. From this point of view, focus should shift from studying single stresses to

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including a combination of different (a)biotic stresses as they truly represent field conditions (Mittler 2006).

ABIOTIC STRESS-INDUCED ROS ACCUMULATION IN PLANTS

Organelles with a highly oxidising metabolism or marked electron flow are a major source of ROS in plants (Fig. 1), both under standard and stressed conditions. In chloroplast thylakoids, the reaction centres of photosystem I (PSI) and photosystem II (PSII) account for a large share of total ROS levels in plant cells (Asada 2006). Abiotic stresses such as excess light (Takahashi & Murata 2008), drought (Asada 2006; de Carvalho 2008), salinity (Miller *et al.* 2010) and metal exposure (Seth *et al.* 2012) increase chloroplastic ROS production either by excitation or partial reduction of O_2 molecules.

In non-photosynthetic cells, mitochondria constitute the main origin of ROS because of electron leakage at the level of complexes I and III in the respiratory electron transport chain (ETC; Fig. 1; Møller 2001; Rhoads *et al.* 2006; Noctor, De Paepe & Foyer 2007). This too can be enhanced in response to various biotic and abiotic stress conditions as reviewed elsewhere (Miller *et al.* 2010; Keunen *et al.* 2011). Although mitochondria were long considered secondary to chloroplasts as cellular powerhouses with lower ROS levels as compared to other organelles, this view has changed as crosstalk and acclimation between mitochondria and other organelles appears increasingly vital for an integrated cellular energy and redox metabolism (Noctor *et al.* 2007; Suzuki *et al.* 2012).

Similar to chloroplasts and mitochondria, peroxisomes produce ROS as by-products of their physiological oxidative metabolism (Fig. 1). Under excess light conditions, they even

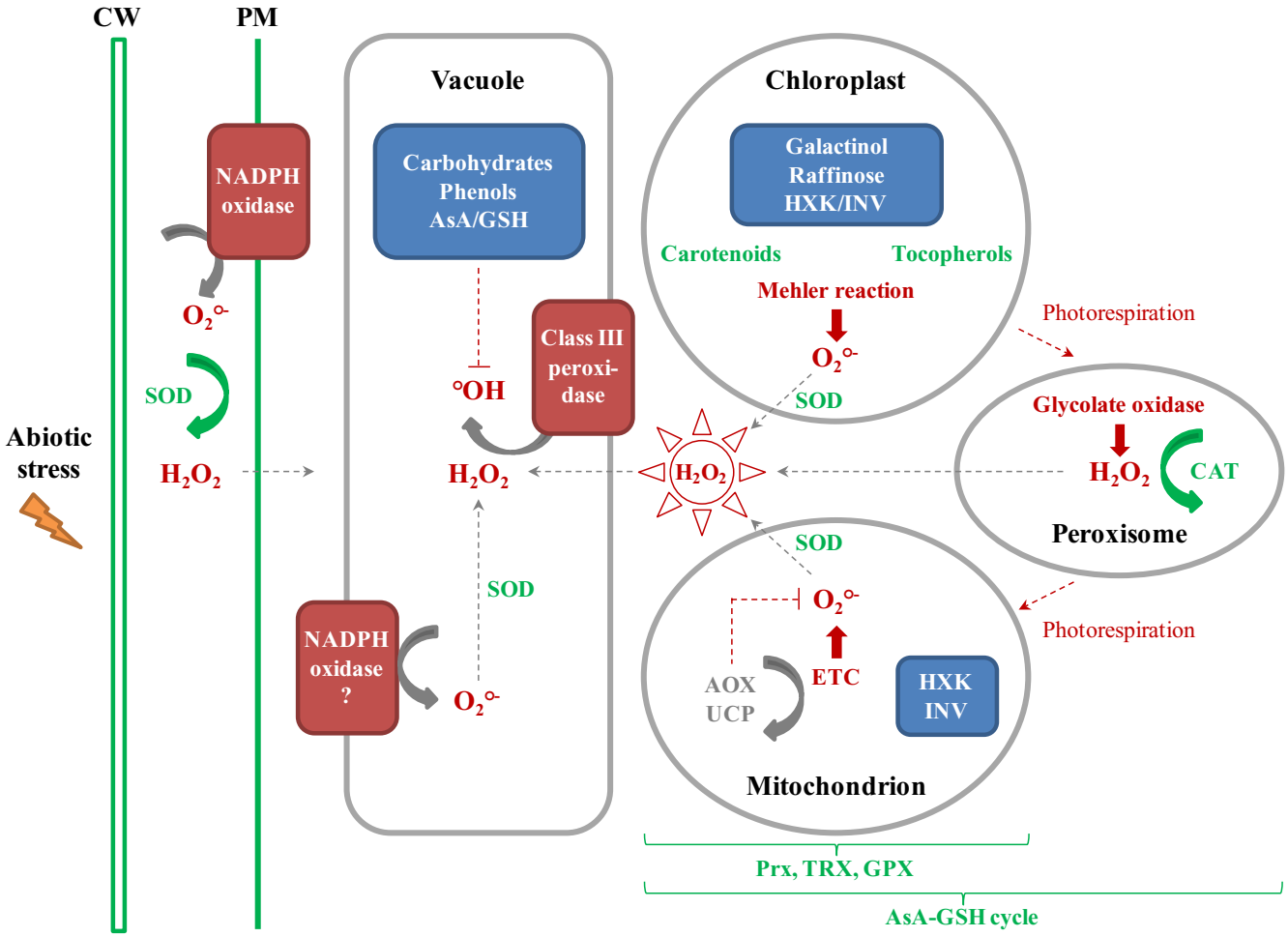

Figure 1. Abiotic stress is commonly leading to an oxidative challenge at organellar and cellular levels, characterised by an imbalance between reactive oxygen species (ROS) production and scavenging via antioxidants in favour of the former. Organelles with a highly oxidising metabolism such as chloroplasts, mitochondria and peroxisomes are renowned for their involvement in stress-induced ROS accumulation. However, the vacuole is increasingly emerging as important contributor via class III peroxidases and potentially NADPH oxidases, similarly as the enzymes involved in ROS production at the plasma membrane. Different organelles seem to exploit different mechanisms to prevent or counterbalance ROS production, in which sugars and their associated metabolic enzymes might play a significant role. AOX, alternative oxidase; AsA, reduced ascorbate; CAT, catalase; GPX, glutathione peroxidase; GSH, reduced glutathione; HXK, hexokinase; INV, invertase; Prx, peroxiredoxin; SOD, superoxide dismutase; TRX, thioredoxin; UCP, uncoupling protein.

constitute the major ROS source concomitantly with chloroplasts (Asada 2006; Van den Ende & Valluru 2009). The photorespiratory pathway is a major producer of H_2O_2 (Foyer & Noctor 2009). In addition, $O_2^{\circ-}$ is produced in the peroxisomal matrix by xanthine oxidase and also in membranes via NAD(P)H-dependent electron transport reactions. Moreover, H_2O_2 is generated by fatty acid β-oxidation, flavin oxidases and dismutation of $O_2^{\circ-}$ (del Río *et al*. 2006). Peroxisomal ROS are implied in stress responses caused by xenobiotics (del Río *et al*. 2002), metals (Rodríguez-Serrano *et al*. 2009) and other (a)biotic stressors such as ozone and soil salinity (del Río *et al*. 2006; Miller *et al*. 2010).

Other – often overlooked – sources of ROS production in plant cells constitute the plasma membrane, cell wall, cytosol, endoplasmic reticulum and vacuole. At the plasma membrane, NADPH oxidases are considered to be the engines of ROS signalling in response to heat, drought, salinity or wounding in plants (Fig. 1; Suzuki *et al*. 2011). The extracellular production of ROS at the cell wall by a pH-dependent cell wall peroxidase could be important during biotic stress (Bolwell & Wojtaszek 1997). In addition, detoxification reactions associated with cytochromes present in the cytosol and endoplasmic reticulum also contribute to ROS production in plant cells (Urban *et al*. 1997; Mittler *et al*. 2004). Lastly, vacuolar ROS production should not be ignored, as several H_2O_2-dependent class III peroxidases are present in this compartment and located at the inner face of the tonoplast (Sottomayor *et al*. 2004; Costa *et al*. 2008). Excess H_2O_2 can enter the vacuole via diffusion through the tonoplast, either directly or facilitated using aquaporins (Bienert *et al*. 2007). Although peroxidases are generally considered to be ROS scavengers, they can also attack H_2O_2, thereby generating highly dangerous $^{\circ}OH$ radicals via the hydroxylic cycle (Passardi, Penel & Dunand 2004; Van den Ende & Valluru 2009 and references therein). Moreover, recent data by Müller *et al*. (2009) suggest that peroxidases – next to NADPH oxidases – are mostly responsible for the production of $^{\circ}OH$ at the cell wall. Therefore, it is tempting to speculate that vacuolar isoforms of these enzymes fulfil similar functions at the tonoplast (Peshev *et al*. 2013). Moreover, proteomic studies have suggested the presence of NADPH oxidases at the tonoplast (Carter *et al*. 2004; Whiteman *et al*. 2008), which might be able to generate $O_2^{\circ-}$ using cytosolic NADPH (Van den Ende & Valluru 2009; Fig. 1).

Next to oxygen-derived reactive intermediates, it becomes increasingly clear that plant cells produce reactive nitrogen species (RNS) such as nitric oxide (NO°) and its related molecules as recently reviewed by Corpas *et al*. (2011). In a broader context, the importance of radicals for all aerobic life forms should be extended to their impact on human health. During the processing of natural products for consumption, food quality decreases because of increased ROS production. Therefore, plant-derived natural antioxidants are marching on as promising food stabilisers (Stoyanova *et al*. 2011). Much research is thus devoted to the potential role of plant compounds combining antioxidative and prebiotic properties, in this way maximising health impact (Van den Ende *et al*. 2011).

ROS: friend or foe?

In plant biology, it is now widely accepted that ROS constitute an ambiguous role during stress responses. Being toxic molecules, they are able to oxidatively injure cells (Møller, Jensen & Hansson 2007). However, they are also key signalling regulators of defence pathways leading to cellular protection and/or acclimation (Mittler *et al*. 2004). The balance between both outcomes is delicate and requires tight control of accumulating ROS levels during abiotic stress in plants (Miller, Shulaev & Mittler 2008; Miller *et al*. 2010).

As $O_2^{\circ-}$ and H_2O_2 are less reactive than $^{\circ}OH$ reacting with every molecule in its neighbourhood within a short half-life of 1 ns (Møller *et al*. 2007), plant cells would benefit from higher concentrations of $^{\circ}OH$ scavengers exactly at positions where $^{\circ}OH$ radicals are potentially generated and could cause substantial damage (e.g. in the vicinity of membranes). Indeed, $^{\circ}OH$ radicals can initiate membrane lipid peroxidation by abstracting a hydrogen atom from the side chain of polyunsaturated fatty acids. This further generates multiple lipid peroxides in a chain reaction, affecting membrane fluidity and functioning (Gill & Tuteja 2010). Lipid peroxidation was shown to occur in plants exposed to a diverse array of abiotic stresses such as salinity (Mittova *et al*. 2003), drought (Vendruscolo *et al*. 2007) and metal exposure (Cuypers *et al*. 2012). As lipid peroxidation is considered as a crucial parameter in selecting plants tolerant to water stress (Sánchez-Rodríguez *et al*. 2010), it is a worthwhile factor to include in studies concerning plant responses and tolerance to abiotic stress.

Both $^{\circ}OH$ and $^1O_2$ are able to attack plant DNA, which finally affects growth and development in various ways (Møller *et al*. 2007). In addition, ROS and/or its by-products are able to covalently modify and oxidise proteins, for which protein carbonylation is a widely accepted marker (Ghezzi & Bonetto 2003). Common protein targets are amino acids containing either sulphur or thiol groups (Gill & Tuteja 2010) and protein oxidation can serve as an alarm signal to initiate or propagate plant responses to abiotic stress (Møller & Kristensen 2004). Therefore, oxidative damage is but one side of the coin.

Conversely, it is generally acknowledged that ROS themselves can act as signalling components mediating plant abiotic stress responses. Next to their versatile properties, mobility and the delicate balance between production and scavenging, they are intimately linked with several signalling and redox networks (Mittler *et al*. 2011). In addition, it becomes increasingly clear that ROS and/or oxidative stress-induced secondary signals are involved in transmitting organelle-specific information to the nucleus during abiotic stress. Galvez-Valdivieso & Mullineaux (2010) recently reviewed the role of ROS in chloroplastic retrograde signalling. Moreover, mitochondrial ROS are suggested to participate in signalling starting from this organelle, especially during metal stress (Yamamoto *et al*. 2002; Rhoads & Subbaiah 2007). Recently, Suzuki *et al*. (2012) reviewed the intense relationship between both organelles in stress-induced redox signalling throughout the

plant cell, emphasising the importance of studying plant responses to abiotic stress simultaneously in different organelles.

THE ROLE OF SUGARS DURING ABIOTIC STRESS

Disaccharides (sucrose, trehalose), raffinose family oligosaccharides (RFOs) and fructans (collectively referred to as 'sugars' from here on) are three major types of water-soluble carbohydrates that are essentially involved in plant stress responses. Sucrose consists of the monosaccharides glucose and fructose and is a widespread disaccharide in nature. As the main product of photosynthesis and its intimate involvement in growth, development, storage, signalling and stress acclimation, it is regarded as the key sugar in plant life (Salerno & Curatti 2003). When this molecule is extended with galactosyl moieties, RFOs such as raffinose, stachyose and verbascose are formed in the cytosol (Schneider & Keller 2009). Next to RFO accumulation (Peters & Keller 2009), the gene expression and activity of enzymes involved in RFO metabolism are highly associated with environmental stress responses (Nishizawa, Yabuta & Shigeoka 2008). Fructans, sucrose-derived fructose polymers assumed to be synthesised in the vacuole, occur in about 15% of all angiosperms. Among these, there are several economically important plants belonging to the *Poaceae*, *Liliales* and *Asterales*, where fructans function as major carbohydrate reserve (Hendry 1993). These plants are crucial in ecosystems that experience frequent environmental changes (Albrecht, Biemelt & Baumgartner 1997). Both RFOs and fructans are sucrosyl oligosaccharides, which – together with their metabolic enzymes – might interact with ROS signalling pathways (Bolouri-Moghaddam *et al*. 2010). The intimate relationship between sugars and ROS will therefore be discussed further on (cfr. *infra*).

The protective role of sugars and their associated metabolic enzymes under stress

Carbohydrates generated by photosynthesis are the building units and energy providers to produce and support plant biomass. In addition, they tightly control transcriptional, posttranscriptional and posttranslational processes *in planta* acting as signalling molecules (Koch 1996; Rolland, Baena-Gonzalez & Sheen 2006; Muller *et al*. 2011). Under mild stress conditions that inhibit plant growth but still allow (partial) photosynthesis, accumulation of disaccharides, RFOs and fructans is commonly observed (Peshev & Van den Ende 2013 and references therein). Recently, Sperdouli & Moustakas (2012) reported an accumulation and interaction of increased proline, anthocyanins and soluble sugars maintaining a high antioxidant protection in *Arabidopsis thaliana* leaves under drought stress. A remodelling of carbon metabolism was also observed in paraquat-exposed *A. thaliana* leaves and interpreted by the authors as an emergency strategy to survive (Scarpeci & Valle 2008). Soil water deficit evokes an increase in soluble carbohydrate concentrations as reported for various plants and sugar compounds (for a review, see Muller *et al*. 2011). By definition, soluble carbohydrates are synthesised in response to osmotic stress, acting as osmoprotectants that stabilise cellular membranes and maintain turgor (Peshev & Van den Ende 2013). In addition, some of them – such as fructans – act as storage carbohydrates (Kawakami, Sato & Yoshida 2008). As an extreme example, resurrection plants use sugar accumulation as one of the mechanisms to cope with complete dehydration (Djilianov *et al*. 2011). Both simple sugars and polysaccharides are able to protect cellular membranes, which is a prerequisite for survival under stress conditions (Valluru & Van den Ende 2008). The responses of transgenic plants carrying extra genes related to sugar metabolism support the protective nature of sugars during abiotic stress (Table 1).

During plant defence responses, signals related to the metabolic enzyme invertase (INV) catalysing sucrose hydrolysis appear to be important as increasing evidence suggests that pathogens alter the plant primary carbohydrate metabolism (Bonfig *et al*. 2010). In addition, extracellular INV is also up-regulated to supply carbohydrates to sink organs during abiotic stress conditions such as salinity (Roitsch *et al*. 2003). The breakdown of cell wall polysaccharides [e.g. oligogalacturonides (Camejo *et al*. 2012)] might therefore also generate sugar signals under stress (Bolouri-Moghaddam *et al*. 2010), which suggests significant overlap and interaction between biotic and abiotic stress responses (Bolouri-Moghaddam & Van den Ende 2012). In addition, it was shown that oligogalacturonides stimulate the enzymatic and metabolic antioxidative defence system in alfalfa roots (Camejo *et al*. 2012), which potentially links sugar signalling with plant defence against oxidative stress (cfr. *infra*).

The intimate relationship between sugars and ROS

Soluble sugars occupy a central position in the cellular redox balance through their close relationships with photosynthesis, mitochondrial respiration and fatty acid β-oxidation (Couée *et al*. 2006). Therefore, variations in sugar levels are able to influence the extent of ROS production in plant cells coupled to the oxidative metabolism in chloroplasts, mitochondria and peroxisomes. In addition, soluble sugars accumulate during different biotic and abiotic stress conditions related to oxidative stress (Couée *et al*. 2006), further pointing towards a relationship between sugars and stress-induced ROS accumulation in plants. For example, it was reported that rice seedlings challenged by chilling, salt and osmotic stress conditions show an enhanced lipid peroxidation and altered carbohydrate metabolism (Morsy *et al*. 2007). It is worthwhile to mention that the observed correlation between sugars and oxidative stress is not a forthright positive one, as it was shown that high sugar levels can either enhance or decrease ROS production in plants. Intriguingly, both high and low sugar levels can evoke ROS accumulation (Couée *et al*. 2006 and references therein). Excess sugar production may result in increased cytosolic H_2O_2 concentrations on the one hand, while sugar availability also

Table 1. Sugar compounds such as disaccharides, RFOs, fructans and sugar alcohols can enhance abiotic stress tolerance in several plant species

Sugar compound	Transgene	Species	Enhanced tolerance to	Reference
Disaccharides				
Trehalose	Trehalose-6-phosphate synthase	*L. esculentum*	Drought, oxidative stress (paraquat or H_2O_2), salinity	Cortina & Culiáñez-Macià 2005
	Trehalose-6-phosphate synthase and phosphatase	*A. thaliana*	Drought, salinity, temperature changes	Miranda *et al.* 2007
	Trehalose phosphorylase	*N. tabacum*	Drought	Han *et al.* 2005
	Trehalose synthase	*N. tabacum*	Drought, salinity	Zhang *et al.* 2005
Raffinose family oligosaccharides (RFOs)				
Galactinol	Galactinol synthase	*A. thaliana*	Oxidative stress (paraquat), chilling, drought, salinity	Nishizawa *et al.* 2008
Raffinose	α-Galactosidase	*Petunia x hybrida cv Mitchell*	Freezing	Pennycooke, Jones & Stushnoff 2003
	Galactinol synthase	*A. thaliana*	Oxidative stress (paraquat), chilling, drought, salinity	Nishizawa *et al.* 2008
	UDP-glucose 4-epimerase	*A. thaliana*	Drought, freezing, salinity	Liu *et al.* 2007
Fructans				
Fructans	Levansucrase	*N. tabacum*	Freezing	Parvanova *et al.* 2004
	Sucrose:sucrose 1-fructosyltransferase	*N. tabacum*	Freezing	Li *et al.* 2007
	Sucrose:sucrose 1-fructosyltransferase and sucrose:fructan 6-fructosyltransferase	*O. sativa*	Chilling	Kawakami *et al.* 2008
Sugar alcohols				
Mannitol	Mannitol-1-phosphate dehydrogenase	*N. tabacum*	Oxidative stress (paraquat)	Shen *et al.* 1997a
	Mannitol-1-phosphate dehydrogenase	*O. sativa*	Drought, salinity	Pujni, Chaudhary & Rajam 2007
	Mannitol-1-phosphate dehydrogenase	*Petunia x hybrida (Hook) Vilm. cv. Mitchell*	Chilling	Chiang, Stushnoff & McSay 2005
	Mannitol-1-phosphate dehydrogenase	*P. taeda*	Salinity	Tang, Peng & Newton 2005
	Mannose 6-phosphate reductase	*A. thaliana*	Salinity	Zhifang & Loescher 2003
Sorbitol	Glucitol-6-phosphate dehydrogenase	*P. taeda*	Salinity	Tang *et al.* 2005
	Sorbitol-6-phosphate dehydrogenase	*D. kaki*	Salinity	Deguchi *et al.* 2004

UDP, uridine diphosphate.

determines the rate of reducing power production contributing to H_2O_2 scavenging by feeding the OPP pathway (Van den Ende & Valluru 2009; Bolouri-Moghaddam *et al*. 2010). The first committed reaction in this pathway is catalysed by glucose 6-phosphate dehydrogenase. Its activity may be crucial in regulating the redox poise and ROS detoxification capacity in chloroplasts (Van den Ende & Valluru 2009 and references therein). In addition, both limited and excess sugars may disturb respiratory metabolism, thereby increasing ROS production at the level of the ETC (Xiang *et al*. 2011).

Small soluble sugars and their metabolic enzymes are presumed to connect to oxidative stress and ROS signalling, but their effects on gene expression are resulting from sugar-specific signalling cascades (Couée *et al*. 2006). Sugar variations are able to modify the expression of genes involved in abiotic stress responses, such as superoxide dismutase (SOD; Koch 1996), heat shock proteins and glutathione-S-transferases (Price *et al*. 2004). Moreover, it was shown that sucrose influences ascorbate (AsA) biosynthesis and recycling in harvested broccoli florets (Nishikawa *et al*. 2005). Until recently, the protective properties of soluble sugars during oxidative stress were therefore generally attributed to direct or indirect signalling triggering the production of ROS scavengers and/or repair enzymes (Van den Ende & Valluru 2009). However, it was recently proposed that sugars might act as true ROS scavengers *in planta*, especially when present at higher concentrations. At low concentrations however, sugars might still function as substrate or signal for stress-induced alterations (Van den Ende & Valluru 2009). This dual role as nutrients and signalling molecules greatly hinders accurate studies of the mechanisms involved, although hexokinase (HXK), Snf1-related kinase 1 and INV are currently identified as conserved sugar signalling components (Valluru & Van den Ende 2011). In addition, sugar signalling is closely interconnected with plant-specific hormone signalling and stress-related pathways (Rolland *et al*. 2006; Bolouri-Moghaddam *et al*. 2010), which again complicates the integrated dissection of abiotic stress-induced signalling mechanisms leading to plant defence responses.

ROS REGULATION BY ANTIOXIDANTS IN DIFFERENT ORGANELLES DURING ABIOTIC STRESS

Enhanced ROS production is generally related to several biotic and abiotic stress conditions, although plant responses differ with respect to the components of the ROS gene network (Mittler *et al*. 2004). Traditionally, plant antioxidants are divided into enzymatic scavengers comprising SOD, ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT), next to non-enzymatic low-molecular weight metabolites such as AsA, glutathione (GSH), α-tocopherol (vitamin E), carotenoids and flavonoids (Fig. 1). Plant abiotic stress tolerance can be enhanced through modification of the expression, content and/or activity of these antioxidants (Gill & Tuteja 2010 and references therein).

Under physiological steady-state conditions, the equilibrium between ROS production and prevention/scavenging is maintained by antioxidative defence components, each confined to a specific compartment of the plant cell (Mittler *et al*. 2004; Miller *et al*. 2010). Up until now, plant antioxidant mechanisms were mainly studied in the cytosol, chloroplasts, mitochondria and peroxisomes. However, recent developments concerning the antioxidant functioning of sugars also point towards antioxidative mechanisms active in plant vacuoles (Van den Ende & Valluru 2009). Therefore, a reconsideration of the classic organellar antioxidant machinery is a major aim of this review, extending the traditional concept with the emerging role of sugars as versatile antioxidants in abiotic stress conditions (Fig. 1).

Sugars as antioxidants in plants

A disturbance of the redox equilibrium in plant cells requires activation of specific antioxidant enzymes neutralising ROS. However, during the initial phase of oxidative stress, low-molecular weight antioxidants play an important role. *In vitro* studies have convincingly demonstrated that the *in vivo* antioxidant capacity of sugars might be highly underestimated. Disaccharides, galactinol, RFOs, fructans and sugar alcohols are proven or suggested to function as antioxidants (Nishizawa *et al*. 2008; Stoyanova *et al*. 2011; Hernandez-Marin & Martínez 2012; Peshev *et al*. 2013). Generally, they are better °OH radical scavengers as compared to $O_2^{\circ-}$ (Stoyanova *et al*. 2011). As no enzymatic °OH scavenging mechanisms exist, plants solely depend on high concentrations of non-enzymatic antioxidants to neutralise these highly dangerous ROS species – next to preventing their formation (Gechev *et al*. 2006). Nishizawa *et al*. (2008) analysed the °OH scavenging ability of galactinol and raffinose *in vitro* and demonstrated similar antioxidant capabilities for both sugars as compared to GSH. In addition, their concentrations are suitably ranged to protect plant cells from oxidative damage (Nishizawa *et al*. 2008). Interestingly, raffinose concentrations in chloroplasts of stressed plants are comparable with those of AsA and GSH, suggesting that this soluble sugar can directly scavenge °OH radicals in chloroplasts (Nishizawa *et al*. 2008; Stoyanova *et al*. 2011).

In vitro studies further demonstrated good ROS scavenging properties of sucrose and fructans (Peshev *et al*. 2013), strongly suggesting that similar reactions might occur *in planta*, especially at higher concentrations (Uemura & Steponkus 2003). Nonetheless, sucrose has not yet been recognised as an antioxidative compound in plants. Van den Ende & Valluru (2009) postulated that this is due to the fact that most research is focussed on *A. thaliana*, containing very low sucrose concentrations unable to elevate under mild stress conditions (Van den Ende & Valluru, unpublished data). However, in other species such as sugar beet and sugar cane and in tissues associated with the phloem, quenching of °OH by sucrose might be of particular importance (Van den Ende & Valluru 2009). This reasoning is supported by the protective effects of exogenous sucrose additions prior or upon exposure to oxidative stress-inducing components such

as herbicides (Ramel *et al*. 2009). Therefore, the classic organellar antioxidant machinery will be discussed in the following paragraphs, with the potential antioxidative functioning of sucrose and other sugars in mind.

Traditional and emerging antioxidants in chloroplasts

Since H_2O_2 is a potent inhibitor of photosynthesis, the chloroplastic redox balance is highly delicate and under tight control of several antioxidant mechanisms recently reviewed by Foyer & Shigeoka (2011) (Fig. 1). Both AsA and GSH are present in a millimolar concentration range and participate in the AsA-GSH cycle within the stroma to neutralise H_2O_2 produced by the disproportionation of $O_2^{\circ-}$ catalysed by SOD. In higher plant chloroplasts, both iron-SOD and copper/zinc-SOD isoforms are present and provide the first line of defence against chloroplastic ROS (Pilon, Ravet & Tapken 2011). Together with APX, these enzymes cooperate in the 'water-water cycle' first described by Asada (1999). This cycle functions both in scavenging of active oxygen molecules and dissipation of excess photons under normal and environmental stress conditions. Moreover, it was recently demonstrated that oxidative stress characterised by increasing H_2O_2 levels drives a significant boost in chloroplastic GSH, again emphasising its critical role during stress conditions (Queval *et al*. 2011; Jozefczak *et al*. 2012).

Next to the AsA-GSH cycle, chloroplasts contain a broad array of enzymatic antioxidants detoxifying photosynthetic ROS. Peroxiredoxins (Prxs) are thiol-based peroxide reductases that reduce several substrates ranging from H_2O_2 to peroxynitrite. Three different types of Prx are present in the chloroplasts of *A. thaliana* and rice, which suggests specific roles in plastid antioxidative defence and signalling (Dietz *et al*. 2006). These enzymes can partly replace APX in a so-called alternative water-water cycle, where they cooperate with GPX to reduce H_2O_2. Consecutively, the oxidised enzymes are reduced by thioredoxins (TRX) or other reductants (Dietz *et al*. 2006). In addition, GPX uses GSH as a reducing substrate and is able to reduce lipid peroxides (Foyer & Shigeoka 2011).

The AsA-GSH cycle components and Prx-dependent pathways scavenge chloroplastic ROS with an equal importance *in vivo* and possible compensation. However, Foyer & Shigeoka (2011) recently argued that their relative importance could vary alongside the environmental conditions at a particular point in time. In addition, the targets of both pathways differ in that the AsA-GSH cycle mainly detoxifies H_2O_2, while Prxs show a broad substrate specificity ranging from lipid peroxides to RNS (Foyer & Shigeoka 2011).

In addition to water-soluble antioxidants, tocopherols and carotenoids are highly abundant hydrophobic antioxidants present in chloroplasts. Tocopherols are present in the envelope and thylakoid membranes, where they efficiently quench $^1O_2$ and specifically scavenge lipid peroxides, also in stress conditions (Maeda & DellaPenna 2007). Much evidence supports a role for chloroplastic tocopherols in mediating abiotic stress responses and potential tolerance (Gill & Tuteja 2010 and references therein). On the other hand, organic carotenoid pigments serve a dual purpose during photosynthesis. Next to light harvesting, they protect the photosynthetic apparatus from photo-oxidation and are generally considered as antioxidants. Therefore, many studies are devoted to altering the carotenoid content and composition in plants, thereby also contributing to a healthy animal diet (Farré *et al*. 2010). Carotenoids have been implicated in the response to several abiotic stressors such as metals (Demirevska-Kepova *et al*. 2006) and ozone exposure in plants (Loreto *et al*. 2004).

Sugars are also able to protect chloroplasts and stabilise photosynthesis in stress conditions. While *in vitro* results clearly demonstrate ROS-scavenging abilities for RFOs (cfr. *supra*), evidence for an antioxidant function mediated by the oligosaccharides galactinol and raffinose *in planta* was provided by the results of Nishizawa *et al*. (2008) using transgenic *A. thaliana* seedlings overexpressing different isoforms of galactinol synthase (GolS1 and GolS2). This enzyme is involved in the biosynthesis of galactinol (from uridine diphosphate-galactose), which is a galactosyl donor to form RFOs such as raffinose. Moreover, its activity is a prerequisite for the accumulation of galactinol and raffinose during environmental stress conditions (Panikulangara *et al*. 2004). Overexpressor seedlings with enhanced GolS activity show higher galactinol and raffinose levels that clearly correlate with their paraquat-tolerant phenotypes. While AsA levels decreased in paraquat-challenged wild-type plants, they remained high in GolS overexpressor seedlings. Moreover, GSH levels were only maintained in stressed overexpressor seedlings and did decrease in the wild type. As both metabolites are able to neutralise oxidising radicals, their maintained concentrations could explain the lower level of lipid peroxidation observed in stressed GolS transgenic plants (Nishizawa *et al*. 2008). These data suggest that galactinol and raffinose protect cellular metabolism and more specifically chloroplastic photosynthesis during paraquat exposure, but also during salinity, chilling or drought stress (Nishizawa *et al*. 2008). Nonetheless, GolS and other RFO-synthesizing enzymes are located outside chloroplasts (Schneider & Keller 2009), which necessitates raffinose import into chloroplasts to fulfil its protective function in these organelles. Recently, Schneider & Keller (2009) demonstrated the presence of raffinose inside chloroplasts of cold-exposed common bugle, spinach and *A. thaliana* plants, where it is transported across the envelope using a raffinose transporter.

Recently, a model depicting the roles of galactinol and RFOs in ROS homeostasis was published by Valluru & Van den Ende (2011), which also emphasises the potential role of neutral INVs and HXK operating in chloroplasts to counteract or even prevent ROS accumulation in stressed plants (cfr. *infra*). In this context, it is worthwhile to mention that oligosaccharides such as galactinol and raffinose differ from traditional antioxidants such as AsA, GSH and tocopherols in that no recycling mechanisms exist to reduce oxidised RFO radicals after they reduced harmful O_2 radicals (Foyer & Shigeoka 2011). Nonetheless, it is suggested that RFO radicals might be regenerated to RFOs using AsA or

other reducing antioxidants such as flavonoids (Bolouri-Moghaddam *et al*. 2010; Peshev & Van den Ende 2013). In addition, these sugars often accumulate to high concentrations in plant cells (Foyer & Shigeoka 2011). When accumulating in the vicinity of thylakoid membranes, they might function as signals or as chloroplastic scavenging antioxidants, but this requires further investigation (Nishizawa *et al*. 2008; Peshev & Van den Ende 2013).

Finally, mannitol is a sugar alcohol proven to possess ROS scavenging capacity, thereby protecting against oxidation by °OH radicals (Shen, Jensen & Bohnert 1997a,b; Stoyanova *et al*. 2011). This compound is believed to protect the function of TRX, ferredoxin and GSH in *Nicotiana tabacum*. Genetically modified tobacco plants containing increased mannitol in their chloroplasts are more tolerant to paraquat as compared to wild-type seedlings, although the actual rate of °OH production did not differ. However, the capacity to scavenge these radicals did increase in transgenic seedlings without any negative impact on photosynthesis, contrary to the effects of other sugars such as glucose, fructose and sucrose (Shen *et al*. 1997a,b; Bolouri-Moghaddam *et al*. 2010).

Prevention of ROS formation in plant mitochondria

As a first line of defence, plant mitochondria contain proactive energy-dissipating systems able to significantly decrease the rate of mitochondrial ROS production at the level of the ETC by maintaining a low ubiquinone reduction level. In this context, the operation of the alternative oxidase (AOX) bypassing respiratory complexes III and IV is proven to diminish ROS production (Møller 2001). Recently, Cvetkovska & Vanlerberghe (2012) established for the first time that a lack of AOX increases steady state *in planta* mitochondrial $O_2^{\circ-}$ concentrations in tobacco leaves. The absence of this enzyme alters stress defences under both normal and stress conditions as reviewed by Van Aken *et al*. (2009). For example, specific antioxidant enzymes such as CAT and GPX were more abundant in tobacco suspension cell cultures or leaves lacking functional AOX, paradoxically leading to lower basal ROS levels as compared to wild-type tissues (Amirsadeghi *et al*. 2006). In addition, it is extensively reported that AOX expression responds to a broad range of biotic and abiotic stressors, suggesting that it is a general target of different stress factors (Van Aken *et al*. 2009; Keunen *et al*. 2011).

Not only is AOX implicated in mediating mitochondrial ROS production, it also is able to dissipate excess reducing equivalents derived from chloroplasts in *A. thaliana*. Thereby, this mitochondrial enzyme effectively supports efficient photosynthesis in chloroplasts (Yoshida, Terashima & Noguchi 2007). By functioning as a sink for excess chloroplastic reducing equivalents, the AOX pathway prevents a build-up of NADPH in the chloroplast stroma, which might otherwise evoke increased ROS generation by over-reduction on the PSI acceptor side and over-excitation of the PSII reaction centres. Thus, by preventing the production of ROS at the level of the photosynthetic ETC, the AOX pathway protects plants against photoinhibition because of increased repair of the photodamaged PSII in *Rumex* K-1 leaves (Zhang *et al*. 2011). Therefore, the classification of AOX as part of the reactive oxygen gene network in plants by Mittler *et al*. (2004) is justified.

Plant uncoupling proteins (UCPs) constitute a second energy-dissipating system that fine-tunes the membrane potential of plant mitochondria, thereby reducing the rate of ROS production in these organelles (Nogueira, Sassaki & Maia 2011). Several studies suggest a role for UCPs during oxidative stress in plants, as their activity is enhanced by $O_2^{\circ-}$ and lipid peroxidation products (Keunen *et al*. 2011 and references therein; Nogueira *et al*. 2011). However, one should always keep in mind that AOX and UCPs are unable to prevent mitochondrial oxidative damage due to ROS diffusing from the cytosol into mitochondria (Navrot *et al*. 2007).

Similarly to AOX and UCPs, sugars and their associated metabolic enzymes could also be involved in avoiding ROS production at the level of the mitochondrial ETC. Bolouri-Moghaddam *et al*. (2010) recently summarised a hypothetical sugar-antioxidant network operating in plant cells, with a central role appointed to glucose and HXK activity, that is predominantly associated with mitochondria. Firstly, by generating glucose-6-phosphate, HXK stimulates AsA biosynthesis via the Smirnoff-Wheeler pathway (Linster *et al*. 2008), thereby contributing to organellar and even cytosolic ROS detoxification. Secondly, a scenario in which the catalytic activity of mitochondria-associated HXK (mtHXK) regulates ROS levels and signalling pathways inducing antioxidant defence systems is now emerging for plants as described for animals (da-Silva *et al*. 2004). Here, mtHXK controls the flux through the mitochondrial ETC via adenosine diphosphate recycling, which supports oxidative phosphorylation and ultimately limits the level of ETC-derived H_2O_2 (Camacho-Pereira *et al*. 2009; Bolouri-Moghaddam *et al*. 2010). Recently, a direct connection between the activity of both mitochondrial and cytosolic alkaline/neutral-INVs (A/N-Invs) and the expression levels of antioxidative defence genes was also suggested using both knockout and overexpressor *A. thaliana* seedlings (Xiang *et al*. 2011). These sucrose-catabolizing A/N-Invs deliver the glucose substrate for HXK and can hereby contribute to mitochondrial and cytosolic ROS homeostasis. In addition, the activity of A/N-Inv enzymes is inhibited by their own hexose products, thereby providing a way to synchronise their activity with that of mtHXK (Xiang *et al*. 2011). In potato tuber mitochondria, similarities between the inhibition of H_2O_2 release by both mtHXK and UCP activity were demonstrated. Which preventive mechanism would prevail then depends on the metabolic conditions, either fuelled by hexoses or β-oxidation (Camacho-Pereira *et al*. 2009). Moreover, Xiang *et al*. (2011) suggested that similar mechanisms using chloroplastic HXKs and A/N-Invs might be operational in chloroplasts, a possibility that was recently reviewed by Valluru & Van den Ende (2011). Interestingly, HXK activity increased in roots and shoots of cadmium (Cd)-exposed pea seedlings,

again supporting a vital role for this enzyme during abiotic stress defence (Devi *et al*. 2007).

ROS scavenging in plant mitochondria

Although avoidance mechanisms are present, $O_2^{\circ-}$ radicals are still produced at the level of the mitochondrial ETC and second-line defence is indispensable (Fig. 1). The first enzyme involved in $O_2^{\circ-}$ detoxification is SOD, which uses manganese (MnSOD) as cofactor to scavenge $O_2^{\circ-}$ in the mitochondrial matrix (Møller 2001). This reaction leads to increased production of H_2O_2 that must be scavenged using other matrix enzymes. As in chloroplasts, the AsA-GSH cycle is fully present and functional in plant mitochondria to neutralise H_2O_2 (Møller 2001; Miller *et al*. 2010). In addition, several enzymes of this cycle are dually targeted to both organelles (Chew, Whelan & Millar 2003), again emphasising their critical role in maintaining organellar and even cellular redox balance. Interestingly, AsA is bound to enter mitochondria in its oxidised form, which depends on a facilitated transport with glucose (Szarka *et al*. 2004). In addition, the last step in AsA biosynthesis is catalysed by the galactono-γ-lactone dehydrogenase enzyme present in the inner mitochondrial membrane (Bartoli, Pastori & Foyer 2000), pointing towards a central mediating role for plant mitochondria in the cellular antioxidant defence by maintaining AsA synthesis (Rhoads *et al*. 2006). Furthermore, plant cells were shown to contain a GPX co-localising within both mitochondria and chloroplasts in poplar, which responded to several biotic and abiotic stressors such as fungal infection, drought stress and metal exposure (Navrot *et al*. 2006). Moreover, subcellular concentrations of GSH were shown to be highest in plant mitochondria and not in plastids as estimated by immunogold labelling densities (Zechmann *et al*. 2008; Queval *et al*. 2011). High and constant levels of GSH in plant mitochondria could contribute to cell survival during abiotic stress, as this metabolite protects mitochondrial DNA and proteins from being oxidised (Zechmann *et al*. 2008 and references therein).

All eukaryotic organisms probably contain at least one mitochondrial Prx isoform, with PrxII F targeted to higher plant mitochondria and able to reduce H_2O_2 (Dietz *et al*. 2006). Using knockout *A. thaliana* seedlings, a crucial role was appointed to this mitochondrial isoform in maintaining redox homeostasis at the cellular level. In addition, a lack of this enzyme leads to severe growth defects under oxidative stress conditions induced by Cd exposure (Finkemeier *et al*. 2005). A strikingly opposite regulation was observed during biotic stress in *Phytophthora infestans*-inoculated *A. thaliana* seedlings, where plastid Prx transcripts declined and the mitochondrial one increased (Dietz *et al*. 2006). The above-mentioned data highly encourage further research on the function of this mitochondrial enzymatic antioxidant during (a)biotic stress conditions. Plant mitochondria are also equipped with a complete TRX and TRX reductase system (Reichheld *et al*. 2005). Moreover, this system is linked to AOX regulation and activation (Gelhaye *et al*. 2004) and therefore not only associated with ROS scavenging, but additionally with ROS avoidance at the level of the ETC (cfr. *supra*; Møller 2001).

Peroxisomal antioxidative defence systems

Peroxisomes are ubiquitous subcellular one-membrane organelles that contain H_2O_2-producing flavin oxidases and CAT as basic enzymatic components (Fig. 1; del Río *et al*. 2002). A complex battery of antioxidant mechanisms is present in peroxisomes, which stresses their value in the oxidative metabolism of plant cells (del Río *et al*. 2006). As they are able to release ROS and RNS as signalling molecules into the cytosol, they contribute to an integrated communication network between cellular compartments under stress. Moreover, their role is highly significant in plants as they proliferate under both natural and abiotic stress conditions such as herbicide or Cd exposure (del Río *et al*. 2002, 2006 and references therein). Induction or alteration of peroxisomal antioxidant defence systems was reported during salinity stress in tomato (Mittova *et al*. 2003), Cd exposure (Romero-Puertas *et al*. 1999) in pea and in response to chilling and water deficit in rice (Morsy *et al*. 2007).

As discussed before, $O_2^{\circ-}$ radicals are produced at two separate locations in plant peroxisomes, and in at least nine species, the presence of peroxisomal SOD to convert these radicals was demonstrated (del Río *et al*. 2002, 2006). Subsequently, CAT catalyses the dismutation of two H_2O_2 molecules into O_2 and H_2O in a fast, reductant-independent way, although its affinity for H_2O_2 is rather low as compared to APX and Prx (Mhamdi *et al*. 2010). The presence of several CAT genes and isoforms has been extensively studied in higher plants, as these enzymes are crucial for the response of plants in stressed conditions (Gill & Tuteja 2010; Mhamdi *et al*. 2010 and references therein). As suggested by Mhamdi *et al*. (2010), CAT-deficient plants could be exploited in studying the responses to (a)biotic stress and dissecting the interplay between different antioxidative defence mechanisms.

The reductant-independent CAT cooperates with the AsA-GSH cycle, of which several components were demonstrated to be present in the matrix and membrane of plant peroxisomes (del Río *et al*. 2006 and references therein). While peroxisomal GSH concentrations were estimated to be rather low in comparison with other subcellular compartments (Queval *et al*. 2011), AsA levels in peroxisomes are among the highest in plant cells (Zechmann, Stumpe & Mauch 2011). In addition, several studies suggest the presence of Prx in plant peroxisomes (Horling, König & Dietz 2002; Corpas *et al*. 2003), which could additionally reinforce the H_2O_2 scavenging network in these organelles.

Until now, no reports exist that clearly demonstrate the involvement of soluble sugars in peroxisomal antioxidant defence. Nonetheless, their availability is strongly linked with the level of ROS production in these organelles, as sugar starvation has been shown to stimulate peroxisomal β-oxidation at different biological organisation levels (Hooks, Bode & Couée 1995; Contento, Kim & Bassham 2004).

Emerging antioxidative defence systems in plant vacuoles

Up to now, the role of the vacuole in oxidative stress has been ignored in almost all review papers. It has been described as an unrevealed player in the ROS signalling network of plants, with largely unknown ROS producing and scavenging potential (Mittler *et al*. 2004). However, it is important to acknowledge its relatively large size, as it accounts for more than 95% of the cellular volume in various plant cells (Van den Ende & Valluru 2009). Moreover, it shows distinguished structural adaptations under stress conditions, leading to the induction of several defence mechanisms (Valluru *et al*. 2008). It should be noted that plant cell vacuoles differ widely regarding their volume, shape and especially composition and function (Marty 1999). This might influence their ROS scavenging capacity as discussed below.

The presence of powerful antioxidants inside vacuoles contributes to their potential to buffer the cellular redox state (Fig. 1). Flavonoids such as flavonols and anthocyanins accumulate to high concentrations in plant vacuoles, playing versatile roles in plant metabolism (Gill & Tuteja 2010). In *in vitro* antioxidant assays, these components often overrule the antioxidative capacity of primary metabolites such as AsA and α-tocopherol. In addition, they were repeatedly shown to accumulate during several biotic and abiotic stress conditions (Winkel-Shirley 2002). Nonetheless, evidence for a true *in vivo* ROS scavenging function is rather limited and therefore still a matter of controversy (Hernández *et al*. 2009; Agati *et al*. 2012).

Although it has long been known that these low-molecular secondary metabolites are present in plant vacuoles, it was unclear whether AsA and GSH were able to cross the tonoplast membrane and accumulate in these organelles. However, it was recently shown that GSH is able to accumulate in vacuoles in its oxidised form, which was suggested to be part of the general response to H_2O_2 in plants (Queval *et al*. 2011). Nonetheless, which transporters are important in transporting this metabolite from the cytosol into the vacuole and its further fate inside this organelle remains unclear (Queval *et al*. 2011). Using AsA-specific immunogold labelling techniques, this metabolite was also demonstrated to be present in vacuoles, although reported levels were lower as compared to those in other cellular compartments in *A. thaliana* and *N. tabacum*. However, in response to high light, the strongest increase in AsA labelling was observed in vacuoles (Zechmann *et al*. 2011). This opens the window to an improved insight into the potential importance of these organelles in AsA metabolism during stress (Zechmann *et al*. 2011), as previously hypothesised by Takahama (2004) almost a decade ago.

More and more, vacuolar sugars and sugar alcohols come into the picture as crucial new players in oxidative stress defence (Bolouri-Moghaddam *et al*. 2010; Stoyanova *et al*. 2011; Peshev & Van den Ende 2013). At higher concentrations, sucrose might function as an antioxidative compound, for example in the vacuoles of sugar beet and sugar cane plants. In addition, fructans were suggested to be more than just reserve water-soluble oligo- and polysaccharides, acting directly as ROS scavengers in the vicinity of the tonoplast (Peshev & Van den Ende 2013). It is generally acknowledged that fructans can intimately integrate in between the headgroups of the tonoplast and stabilise this membrane during stress conditions such as freezing and drought (Valluru & Van den Ende 2008; Bolouri-Moghaddam *et al*. 2010). Additionally, they are ideally positioned in this way to scavenge any °OH radicals formed in the proximity of the tonoplast by the action of vacuolar oxidases and peroxidases, thereby preventing lipid peroxidation (Van den Ende & Valluru 2009). This reaction leads to the production of H_2O and less damaging fructan radicals, which might be recycled back into fructans using phenolic compounds and/or vacuolar AsA or GSH as reductants (Bolouri-Moghaddam *et al*. 2010; Peshev & Van den Ende 2013), assuming their presence in the vacuole (cfr. *supra*). Phenolic compounds and fructans might therefore operate synergistically to scavenge excess vacuolar H_2O_2 (Bolouri-Moghaddam *et al*. 2010). It should be noted that this model strictly depends on high sucrose concentrations to support fructan biosynthesis by fructosyl transferase enzymes (Van den Ende & Valluru 2009), again emphasising the critical role of sucrose in antioxidative defence.

Experimental evidence for the above-mentioned concepts was recently published by Peshev *et al*. (2013), who studied the *in vitro* °OH scavenging capacities of various vacuolar carbohydrates and phenolic compounds. They revealed that the most effective antioxidants possess a C = C bond in their side chains. Among the tested carbohydrates, the strongest antioxidant properties were observed for the fructan compound inulin. Its °OH scavenging capacity is similar as compared to chicoric acid and even higher than observed for gallic acid (Peshev *et al*. 2013). In addition, the fate of sugars reacting with radicals was discussed, pointing out that all reactions generate a new – probably less reactive – sugar radical as end product. Interestingly, non-enzymatic *de novo* synthesis of fructosyl oligosaccharides based on radical combination was also evidenced (Peshev *et al*. 2013). Gathering evidence for sugars reacting with °OH radicals at the tonoplast *in vivo* harbours experimental challenges, as monitoring carbohydrate breakdown or synthesis also includes changes by the action of endogenous enzyme activities (Peshev *et al*. 2013). Nonetheless, the available *in vitro* evidence opens the window for future studies that will fill the current gap between hypothetical working models and true *in planta* events.

Indirectly, fructans might stimulate other specific antioxidative defence mechanisms. Intriguingly, alterations in fructan concentrations are closely associated with changes in AsA and GSH concentrations in immature wheat kernels (De Gara *et al*. 2003). In addition, the glucose that is formed during fructan synthesis may fuel the biosynthesis of classic antioxidants such as AsA in the cytosol (Bolouri-Moghaddam *et al*. 2010 and references therein). These observations suggest an intimate relationship between cytosolic and vacuolar antioxidative defence mechanisms (Bolouri-Moghaddam *et al*. 2010; Peshev & Van den Ende 2013). Under stress conditions, fructans and sucrose might even be carried from the vacuole to the apoplast via tonoplast

vesicle-derived exocytosis, thereby also stabilising this membrane and contributing to a maintained cellular integrity and survival (Valluru *et al*. 2008; Van den Ende & Valluru 2009).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Worldwide, environmental stress conditions disturb the cellular redox equilibrium of plants, often resulting in increased ROS production. Traditionally, enzymatic and non-enzymatic antioxidants were studied as ultimate defence pathways detoxifying ROS and thereby determining plant responses to abiotic stress. Recently, a concept is emerging in which sugars such as sucrose, RFOs and fructans, known to contribute indirectly to classic antioxidative mechanisms, are also involved in direct ROS quenching in different organelles, thereby contributing to abiotic stress tolerance. Moreover, an interaction between ROS and sugar signalling pathways is to be expected, pointing towards sugars functioning in an integrated cellular redox network. Much is still to be learned about the exact chemical identity and stability of sugar radicals and how they determine the fate of stressed plants. Nonetheless, introducing specific sugars – as osmoprotectants and/or antioxidants – next to modulating the presence and activity of key sugar metabolic enzymes, are promising tools to develop stress tolerant crops with increased yield, quality and longevity under challenging environmental conditions. Moreover, various vacuolar phenolic compounds and sugars are present in medicines or used as food additives. Future studies should therefore focus on these targets – as well as their associated metabolic enzymes – not only to improve stress tolerance in crops, but also to enhance food quality.

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