Chapter 13
Sugars as Antioxidants in Plants

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1 Plant Carbohydrates: An Introduction

Typical plant carbohydrates consist of carbon, hydrogen and oxygen. Their empirical formula is $C_m(H_2O)_n$. (m and $n \geq 3$, $m \geq n$). They are polyhydroxy aldehydes and ketones with different degrees of polymerization (DP). On this basis, monosaccharides (DP1), disaccharides (DP2), oligosaccharides ($DP \leq 10$), and polysaccharides ($DP > 10$) can be distinguished. Some biomolecules do not strictly obey the empirical formula (e.g., chitin), but they are still considered as carbohydrates.

Carbohydrates are the most abundant biomolecules on the planet. They fulfill numerous functions in living beings (He and Liu 2002), especially in plants (Lewis 1984). Polysaccharides are often used to store energy (e.g., starch, glycogen, fructans), or to make up structural components (e.g., cellulose and chitin). Ribose and deoxyribose form an integral part of RNA and DNA. Ribose is also a part of NAD(P)H and ATP which are important cellular metabolites.

Numerous carbohydrates are recognized participants in stress responses in plants and other living beings. This chapter will discuss in detail the relations between carbohydrates and plant stress responses, particularly focusing on their role in the plant antioxidant network. First, the plant stresses are introduced. Second, the metabolism of stress-related sugars is highlighted. Third, the actual functions of different types of sugars in plants will be discussed, with special attention on the emerging "sugar as antioxidant" concept in which sugars act as true ROS scavengers in plants. Finally, how these insights might be exploited to create stress-tolerant crops in the near future is discussed.

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2 Threats in the Field: The ROS-Stress Connection

Plants as sessile organisms are subjected to various forms of environmental stresses such as salinity, UV radiation, drought, heavy metals, temperature variations (heat-shock, chilling, frost), nutrient deficiency, air pollution, herbicides and pathogen attacks, from which they cannot escape. They need to be able to develop defense mechanisms to cope with such unfavorable factors (Nishizawa et al. 2008). It is generally accepted that the imposition of the above mentioned stresses leads to excess concentrations of reactive oxygen species (ROS; Nishizawa et al. 2008). Crop yield and quality are negatively-affected by all these different types of stresses, potentially leading to oxidative damage (Bolouri-Moghaddam et al. 2010). In other words, oxidative damage is likely the main reason for yield and quality losses under stress.

2.1 *ROS Identity, Origin and Function*

In plants, ROS are continuously produced in chloroplasts during the process of photosynthesis, during the respiration process in mitochondria, in peroxisomes and at the plasma membrane (NADPH oxidases). ROS include hydroxyl radicals ($^{\bullet}OH$), superoxide radicals ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Among these, the $^{\bullet}OH$ is the most reactive (*in vivo* half-life 10^{-9} s) and dangerous species, immediately attacking virtually any molecule in its neighbourhood. By contrast, H_2O_2 is much more stable and it is able to cross membranes. To deal with ROS, plants develop mechanisms that keep the equilibrium between the production and the scavenging of ROS in the cell: ROS homeostasis (Gill and Tuteja 2010). In general, two antioxidant protective mechanisms are discriminated: enzymatic and non-enzymatic mechanisms. The former includes the action of superoxide dismutase (SOD), ascorbate (AsA) peroxidase (APX), glutathione (GSH) peroxidase (GPX), thioredoxin peroxidase (TPX) and catalase (CAT) (Nishizawa et al. 2008). Non-enzymatic antioxidants include polyphenols, AsA, GSH, flavonoids, carotenoids, α-tocopherol, sugar-sterols and sugar-phenols and soluble carbohydrates, such as fructans and Raffinose Family Oligosaccharides (RFOs) (Stoyanova et al. 2011).

The ROS equilibrium determines whether it causes damage or rather acts as a signal to induce defense responses under abiotic and biotic stresses (Gill and Tuteja 2010). Therefore, spatio-temporal variations in ROS are greatly important to understand whether ROS (e.g., H_2O_2) could act as a signal or not (Gill and Tuteja 2010). ROS levels likely differ among different plant organelles, being intimately linked to the specific metabolism and antioxidant mechanisms taking place at these different locations. Under stress, the delicate balance is disturbed leading to temporal ROS accumulation (ROS overshoot) in one or more organelles, and finally also in the whole cell, including the cytosol, nucleus and the vacuole (see below; Gill and Tuteja 2010). This might lead to oxidative damage of biomolecules, including nucleic acids, proteins and lipids (Gill and Tuteja 2010; Nishizawa et al. 2008).

2.2 *Abiotic Threats and Their Connection with ROS*

Often, plants are challenged with different types of stresses at the same time. Such combined stresses can cause severe harm or decrease a plant's ability to resist consequential stresses (Tester and Bacic 2005). For example, low water supply is often accompanied by high temperature stress, high photon irradiance and soil mineral toxicities constraining root growth (Tester and Bacic 2005). More severe drought stresses can make a plant more susceptible to damage from high irradiance (Tester and Basic 2005). Another example is chilling stress going along with water deficits by disturbed water transport, leading to ROS accumulation and damage to cell membranes (Hodges et al. 1997; Kawakami et al. 2008). In fact, the first harmful effects in case of hypothermia in cold-tolerant and, especially, cold-sensitive plants are caused by increased oxidative stress. The generation of the superoxide radical seems to occur in an initial step (Sinkevich et al. 2010).

Other stress conditions also lead to increased ROS production, such as high photosynthetic activity in source leaves leading to temporal sugar accumulation. However, these sugars are believed to counteract (directly or indirectly) oxidative stress, perhaps contributing to re-establishing cellular ROS homeostasis (see below). Sugar starvation too can lead to ROS accumulation (Bolouri-Moghaddam et al. 2010). Taken together, both sugar shortage and excesses might lead to a disturbance of respiratory metabolism, leading to excess ROS during mitochondrial electron transport (Xiang et al. 2011). In rice, cytoplasmic male sterility (CMS) was found to be correlated with ROS overproduction and ATP depletion, leading to mitochondrial failure and disturbance of pollen development (Nguyen et al. 2010).

3 Stress-Related Carbohydrates and Their Metabolism

Some soluble carbohydrates (glucose, fructose and sucrose) in concert with hormone-signaling pathways are crucial in signaling events controlling plant growth and development (Smeekens et al. 2010; see also below). There are three main water-soluble carbohydrate types (collectively referred to as "sugars" from this point on) that play essential roles in plant stress responses: disaccharides (sucrose, trehalose), RFOs and fructans. Typically, such sugars can accumulate under (mild) stresses when growth is restricted but photosynthesis is not (or only partially) inhibited (De Roover et al. 2000; Muller et al. 2011; Skirycz et al. 2011).

3.1 *Disaccharides*

Sucrose (Glcα(alfa)1,2β(beta)Fru) is one of the most widespread disaccharides in nature (Salerno and Curatti 2003). In higher plants, it represents the major transport compound bringing carbon skeletons from source (photosynthetically active leaves) to sink tissues (roots, young leaves, flowers, seeds, etc.)(Koch 2004). Although sucrose is a major reserve compound in some plants (e.g., sugar beet, sugarcane), it should be noted that starch is the most prominent reserve carbohydrate in most plants. It is a glucose polymer consisting of linear amylose (α(alfa)1,4 linkages) and branched amylopectin chains (α(alfa)1,4 and α(alfa)1,6 linkages). In contrast to sucrose which only occurs in plants and some algae, trehalose (Glcα(alfa)1,1Glc) is found in all domains of the tree of life. It accumulates for instance in fungi and in insects (Elbein et al. 2003; Muller et al. 1995). Upon drying, only a few so-called resurrection plants are known to accumulate trehalose to a great extent. Most higher plants contain different trehalases that prevent trehalose accumulation (Muller et al. 1995), but they keep on synthesizing low amounts of trehalose 6-phosphate (T6P via Trehalose 6-Phosphate Synthase) and trehalose (via Trehalose 6-Phosphate Phosphatase), the former most probably involved in sugar signaling (Zhang et al. 2009; see also below).

3.2 Raffinose Family of Oligosaccharides

Raffinose family of oligosaccharides (RFOs) are α(alfa)(1,6) galactosyl extensions of sucrose, such as raffinose (DP3), stachyose (DP4) and verbascose (DP5). Raffinose is nearly ubiquitous in plants (Vanhaecke et al. 2008). Lychnose and its derivatives can be considered as alternative RFOs, derived from raffinose (Vanhaecke et al. 2010). The metabolism of classic RFOs is well known. First, galactinol synthase (GolS) synthesizes galactinol from UDPGal and myoinositol. Next, raffinose synthase (RafS) transfers the galactose residue to sucrose to form raffinose. Stachyose synthase (StaS) fulfils a similar reaction with raffinose as acceptor substrate. With the synthesis of higher DP (>DP4), RFOs occur independent of galactinol. Galactan:galactan galactosyl transferases (GGTs) are used for this purpose (Tapernoux-Luthi et al. 2004). Raffinose and stachyose are synthesized in the cytoplasm (Schneider and Keller 2009). Recently, it has been reported that RFO gene expression and enzymatic activities and RFO accumulation are closely associated with responses to environmental stress (Nishizawa et al. 2008; Peters and Keller 2009). RFO biosynthesis in *Arabidopsis* seems to be mainly regulated at the level of transcription (Espinoza et al. 2010). Stachyose typically accumulates in *Arabidopsis* seeds (Taji et al. 2002). RFOs play a main role in many other seeds too (Blochl et al. 2008).

3.3 Fructans

Fructans are water-soluble sucrose-derived fructose polymers accumulating in about 15 % of the angiosperm flora (Hendry 1993), but they also occur in a wide range of bacteria and in some fungi. Some economically-important plant groups are known to accumulate fructans, such as *Poales*, *Liliales* and *Asterales* (Hendry

1993). The fructan syndrome probably arose some 30 ± 15 million years ago during a climatologic shift to seasonal drought (Hendry 1993). Thus, drought might have been an important evolutionary trigger and fructans may have played a role in drought resistance (De Roover et al. 2000). Plants with the ability to synthesize fructans are important components of ecosystems that experience frequent environmental changes (Albrecht et al. 1997). Typically, the DP of fructans is modified during such changes (Amiard et al. 2003).

The fructans have been classified in five different types depending on the type of linkages between the fructosyl units and their branching: Inulin consisting of β(beta)(2,1) linkages, levan containing β(beta)(2,6) linkages and graminan having both β(beta)(2,1) and β(beta)(2,6) linkages. Neo-inulin and neo-levan type of fructans as occurring in *Lolium*, *Asparagus* and *Allium* contain an internal glucose residue (Van den Ende et al. 2002). Fructans are believed to be synthesized in the central vacuole. The first step in fructan synthesis is catalyzed by sucrose: sucrose 1-fructosyltransferase (1-SST), synthesizing glucose and 1-kestotriose from two sucrose molecules. Depending on the species, further elongation occurs through the action of a specific number of other fructosyltransferases (FTs; 1-FFT, 6G-FFT and 6-SFT) by further adding β(beta)(2,1)- and/or β(beta)(2,6)-linked fructosyl moieties (Van den Ende et al. 2011 and references therein). Fructan breakdown is accomplished by fructan exohydrolases (FEHs). 1-FEH, 6-FEH, 6-KEH (6-kestose exohydrolase), and 6&1-FEH type of enzymes have been described in fructan plants (Kawakami et al. 2005 and references therein). However, such FEHs also occur in non-fructan accumulating plants but their functions in such plants remain unclear (De Coninck et al. 2005). The regulation of fructan biosynthetic and breakdown genes is mainly controlled at the transcriptional level (Van Laere and Van den Ende 2002). Dicot FT genes are specifically induced by sucrose and dicot FEH genes are induced by cold (Michiels et al. 2004). However, many FEHs are also inhibited by sucrose at the posttranslational level (Van den Ende et al. 2001). Bacterial fructans (levans, inulins) are much longer than plant fructans and involve the activity of levansucrases and inulosucrases (Banguela et al. 2011).

Fructans can act as long-term (inulins, dicots) and as short-term (other fructan types, grasses) reserve carbohydrates in different organs such as roots, stems, grains and sometimes in leaves (Pollock and Cairns 1991; Van Laere and Van den Ende 2002). Similar to invertases splitting sucrose into glucose and fructose (Koch 2004; Roitsch and Gonzalez 2004), the sucrose splitting capacities of some FTs can be used to establish sucrose gradients and regulate sink strength (Ji et al. 2010). It can be speculated that storing fructans might be advantageous compared to starch. First, FTs are less inhibited by cold compared to starch synthases (Pollock et al. 1999). Second, remobilization of water soluble fructans is likely to occur at a faster rate compared to the insoluble starch (Van Laere and Van den Ende 2002).

Furthermore, fructans and FEH regulate osmosis during flower opening in *Campanula rapunculoides* and other species (Vergauwen et al. 2000). Fructans fuel rapid regrowth in grasses and act as membrane stabilizers under stress (Valluru and Van den Ende 2008; Lothier et al. 2010).

Inulin-type fructans, derived from chicory, are widely used as prebiotics in functional food leading to improved health and well-being (Roberfroid 2007). Recent insights suggest that they could counteract oxidative stress in the human body as well (Stoyanova et al. 2011).

4 Sugars as Osmoprotectants

One of the mechanisms that plants use to combat the detrimental effects of environmental stress is to synthesise different kinds of protective compounds such as compatible solutes and antioxidants. Soluble carbohydrates (e.g., trehalose, sucrose, raffinose and fructans) along with certain amino acids (e.g., proline), quaternary ammonium compounds (e.g., glycinebetaine), and polyols (e.g., mannitol) are thought to be compatible solutes. They are synthesized in response to osmotic stress by definition and can occur at high intracellular concentrations without interfering with normal cellular metabolism (Shen et al. 1997; Mundree et al. 2002; Parvanova et al. 2004). They act as osmoprotectants and some of them (e.g. fructans) are also storage carbohydrates (Kawakami et al. 2008). Compatible solutes facilitate osmotic adjustments during water stress and in addition may serve as protective agents by stabilizing proteins and membranes (Hincha et al. 2002). It should be noted that most compatible solutes show excellent capabilities to scavenge ROS *in vitro,* suggesting that they have similar roles in plants (Van den Ende and Valluru 2009; see also below).

4.1 General Mode of Action under Stress

The primary cause of injury during freezing is the destabilization of cellular membranes (Uemera and Steponkus 1999). Also during desiccation, the cell needs to keep all its membranes (plasma membrane, tonoplast membrane, organellar membranes) and proteins in proper functional state. In general, soluble sugar levels contribute to the increased cryostability of cellular membranes (Ma et al. 2009), keeping membranes in their proper state which is a prerequisite for survival under unfavorable conditions. Resurrection plants develop an array of mechanisms to survive complete dehydration, sugar accumulation being one of them (Djilianov et al. 2011). Sugars can replace water under drought stress. As such, they keep membrane surfaces "hydrated" and prevent membrane fusion by maintaining the space between phospholipid molecules (Mundree et al. 2002; Valluru and Van den Ende 2008). This can be explained in terms of 'sugar vitrification'. It includes the formation of a solid, amorphous glass that prevents membrane fusion. Hydrogen bonds are not present in such glassy states. The magnitude of sugar vitrification depends on the temperature at which the glass devitrifies (T_g), which itself depends on the molecular weight of the sugar and on the water content (Levine and Slade 1991).

The model of Wolfe and Bryant (1999) strongly suggests that sugar vitrification is not only necessary but sufficient for the preservation of a dry membrane (Valluru and Van den Ende 2008). In summary, sugars may contribute to an efficient membrane protection in the dry state because of reducing the phase transition temperature (T_m) and forming an amorphous carbohydrate glass with high melting temperature T_g (Valluru and Van den Ende 2008). Both may operate in parallel. The ratio of about 1.5 glucose/sucrose per lipid molecule is thought to be optimal for membrane protection (Lenne et al. 2007) and was also found effective in reducing the T_m. This is supported with data obtained for trehalose. More details are described in Valluru and Van den Ende (2008).

Sucrose is thought to function as a typical osmoprotectant, stabilizing cellular membranes and maintaining turgor. As an easily metabolisable sugar, sucrose may serve as an immediate energy source upon rehydration (Mundree et al. 2002). In most cases (excluding trehalose type of resurrection species), sucrose accumulation is observed under drought stress, albeit at varying levels among species, implicating a role for sucrose in the acquisition of desiccation tolerance in these plants. Many kinds of soluble, sucrose-derived sugars can arise during the course of cold acclimation (see also below: RFOs in *Arabidopsis*). For instance, some mosses accumulate such soluble sugars (Nagao et al. 2006) in association with development of freezing tolerance. Also woody plants (mainly RFO accumulators) and cereals (mainly fructan accumulators) are known to develop high degrees of freezing tolerance in winter, associated with the increased levels of such compounds during cold acclimation (Sauter et al. 1996; Livingston et al. 2009). It is also proposed that the role of some oligosaccharides (e.g., raffinose) is to prevent crystallization of sucrose (Peters et al. 2007). Therefore, the sucrose/raffinose ratio might be more important than the absolute sucrose and raffinose concentrations as such (Djilianov et al. 2011).

In the following two sections, we will focus on transgenic plants carrying extra genes related to RFO and fructan metabolism. In general, the reactions of these plants under stress corroborate the protective nature of these sugars.

4.2 RFOs in Transgenic Arabidopsis

As already indicated above, RFOs are implicated in freezing tolerance in the model plant *Arabidopsis thaliana*. Both galactinol and raffinose increase in stressed *Arabidopsis* plants (Taji et al. 2002). Accordingly, downregulation of an α(alfa)-galactosidase in *Petunia* resulted in increased raffinose levels and cold tolerance (Pennycooke et al. 2003). *Arabidopsis* contains seven GolS genes, one of which is induced by cold (GolS3), two other ones by drought (GolS1 and GolS2; Taji et al. 2002). Two ecotypes with different freezing tolerance (C24 and Columbia) also showed clearly different raffinose levels, suggesting a positive correlation between raffinose levels and freezing tolerance (Klotke et al. 2004). However, *Arabidopsis* RafS knockout plants affected in raffinose synthesis were not more sensitive to frost, suggesting that the increased galactinol levels in these lines could compensate for the impaired raffinose levels (Korn et al. 2010).

Overexpression of drought-inducible GolS1 and GolS2 genes in *Arabidopsis* also led to increased galactinol and raffinose levels, and showed reduced transpiration from leaves to improve drought tolerance (Taji et al. 2002) . Moreover, the intracellular levels of galactinol and raffinose in these transgenic plants were correlated with increased tolerance to paraquat treatment and salinity or chilling (Nishizawa et al. 2008). However, the debate is still on over the exact mechanism of action of these metabolites. They might act as signals but more likely they act as true ROS scavengers when they accumulate at high concentrations at particular locations (e.g., in the vicinity of chloroplast thylakoid membranes; Foyer and Shigeoka 2011). Intriguingly, introduction of a StaS from adzuki bean in *Arabidopsis* did not lead to increased freezing tolerance (Iftime et al. 2011). Probably stachyose is not at the correct place (cytosol) to provide protection. It can be speculated that *Arabidopsis* lacks a stachyose transporter in the chloroplastic envelope. However, recent evidence was generated for the presence of a raffinose transporter in the chloroplastic envelope (Schneider and Keller 2009).

4.3 Transgenic Fructan Plants

The reviews of Cairns (2003) and Banguela and Hernández (2006) summarize the efforts to introduce fructan metabolism in (mostly) non-fructan accumulating species. In a first phase, focus was on introducing bacterial levansucrases in such species (Cairns 2003). However, much debate arose on the correct delivery of these levansucrases to the vacuole and to plastids (mistargeting to the ER; putative ER contamination in plastid preparations, etc.). In many cases, only low levels of fructan were found (max 10 % on dry weight basis) since higher levels appeared to become toxic for these plants (Cairns 2003). Recently, Banguela et al. (2011) fused the preproprotein of onion 1-SST to the levansucrase of *Gluconacetobacter diazotrophicus* and introduced this in tobacco, in order to obtain a correct vacuolar delivery. Some transgenic lines accumulated levans up to 70 % on dry weight basis, and they showed phenotypic changes (leaf bleaching) during plant development. However, only slight leaf bleaching was observed in plants accumulating up to 30 % levans on dry weight basis (Banguela et al. 2011).

In the second phase, interest shifted to introducing plant FTs in non-fructan plants (Sevenier et al. 1998; Hellwege et al. 2000; Stoop et al. 2007; Li et al. 2007; Kawakami et al. 2008; Pan et al. 2009) or in plants accumulating other types of fructans (Vijn et al. 1997; Hisano et al. 2004; Gadegaard et al. 2008). In most cases, no detrimental effects on the plants phenotype were reported in these experiments. This might be explained by the correct vacuolar targeting of these enzymes and, perhaps, by the presence of endogenous FEHs being able to degrade the fructans at specific phases of plant development (e.g., pre-flowering stages).

More importantly, for some of these fructan accumulating transgenic plants, it was reported that they were more tolerant to stress. Konstantinova et al. (2002) showed that transgenic tobacco carrying the *Bacillus subtilis* levansucrase gene Sac B survived freezing stress both in controlled and in field conditions. Transgenic *Lolium* plants carrying wheat FT genes (1-SST or 6-SFT) and accumulating increased amounts of fructan also demonstrated enhanced freezing resistance at the cellular level (Hisano et al. 2004). Rice (*Oryza sativa* L.) is a non-fructan accumulating plant which is highly sensitive to chilling (Kawakami et al. 2008). Transgenic seedlings carrying wheat 1-SST and accumulating fructan oligo- and polysaccharides showed enhanced chilling tolerance. Introduction of the 1-SST of lettuce (*Lactuca sativa* L; Li et al. 2007) also led to increased freezing tolerance and reduced oxidation of membranes, similarly as observed in the *Arabidopsis* GolS overexpression plants (Nishizawa et al. 2008). Taken together, these data strongly suggest that sugars can contribute to stress tolerance by protecting membranes.

4.4 Fructan-Membrane Interactions

Sucrose and trehalose are well-known membrane stabilizers, and also fructans and RFOs have such properties, while hydroxyethyl starch, glucan and dextran have not. It is believed that fructans can replace water molecules at the membrane. Sucrose and trehalose can replace around 18 water molecules while 1-kestose has a volume equal to about 21 water molecules. For raffinose, this number is about 30 (Valluru and Van den Ende 2008). The Fru-Fru linkage (CH_2-O) in fructans is longer than the O-linkage in hydroxyethyl starch, glucan and dextran, creating an extra flexibility to interact with and stabilize membranes (Valluru and Van den Ende 2008). Inulin chains are even more flexible than levans (Vereyken et al. 2003). Using liposomes as a model system, five fructan classes (DP3, DP4, DP5, DP6 and DP7) and two DP>7 fractions were isolated from oat and rye and tested as membrane stabilizators *in vitro* (Hincha et al. 2007). The two DP>7 fractions from both species were unable to protect liposomes, while the fractions containing smaller fructans were protective to different degrees. Protection showed an optimum at DP4. Intriguingly, synergistic effects were found when low DP fructans were combined with DP>7 fructans, suggesting that mixtures of fructans, as they occur in living cells, may have protective properties that differ significantly from those of the purified fractions. However, no mechanistic insights are yet available to explain these observations. Accordingly, the capacity to accumulate higher DP fructans has been found in many stress-tolerant species, such as *Echinops*, *Viguiera*, *Dactylis*, *Lolium*, *Poa* and *Pachysandra* (Van den Ende et al. 2011 and references therein).

Next to protecting the tonoplast, fructans might also protect the plasma membrane. Tonoplast vesicle-derived exocytosis (TVE, see also below in Fig. 13.1) was proposed as a mechanism to transport fructans from the vacuole to the apoplast under stress (Valluru et al. 2008). No fructan transporters have yet been reported in

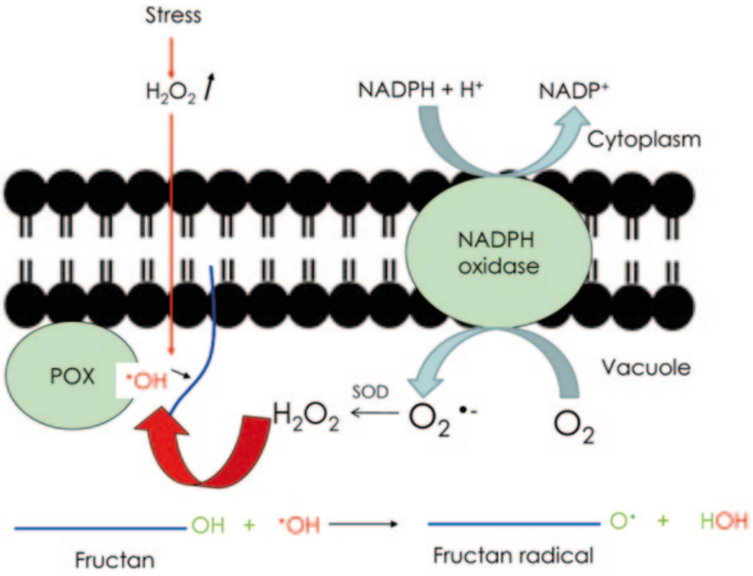

Fig. 13.1 A dual role for vacuolar fructans in the vicinity of the tonoplast under stress? Abiotic and biotic stresses can lead to increased concentrations of cytosolic H_2O_2, which can enter the vacuole via diffusion and/or through aquaporins. Alternatively, the oxidative stress can be transmitted through the action of a putative vacuolar NADPH oxidase. Vacuolar fructans can insert deeply between the headgroups of the tonoplastic membranes, stabilizing them under stress. Type III peroxidases (POX) associate intimately with the inner side of the tonoplast. Peroxidases also produce $^{\bullet}$OH radicals. Fructans are well-positioned to scavenge these radicals, a process in which fructan radicals and water are formed. Fructan radicals might be generated back into fructans with the help of phenolic compounds. (see Fig. 13.2)

the chloroplast envelope. Yet, the finding of raffinose transporters at this location urges further research in fructan accumulating plants.

5 Sugars as Signals

Small soluble sugars (glucose, fructose, sucrose) can also act as signals. They are now recognized as pivotal integrating regulatory molecules that control gene expression related to plant metabolism, stress resistance, growth and development (Rolland et al. 2006; Smeekens et al. 2010; Cho and Yoo 2011; Li et al. 2011). It is becoming increasingly clear that the hexose/sucrose ratio is an important parameter to adjust plant metabolism (Weber et al. 1995; Xiang et al. 2011). Therefore, the interest in acid and neutral type of invertases and their inhibitors (or binding partners) is steeply increasing (Xiang et al. 2011; Hothorn et al. 2010). Intriguingly, the responses to sugar signals and to oxidative stress are linked. Sugars also affect scores of stress-responsive genes (Bolouri-Moghaddam et al. 2010) but the crosstalk between sugar and ROS signaling pathways needs further exploration.

Moreover, many jasmonate-, ABA-, and stress-inducible genes are coregulated by sugars (Ma et al. 2009).

So far, hexokinase (HXK1) and Snf1-related kinase 1 (SnRK1) have been identified as conserved sugar signaling components controlling energy homeostasis, stress resistance, survival and longevity (Moore et al. 2003; Baena-Gonzalez et al. 2007). Both glucose and HXK (producing glucose 6-phosphate) take a central position in sugar signaling and antioxidant networks. They form an important bridge between sugar metabolism and biosynthesis of AsA and of glycosylated phenolic compounds via NDP-glucose production (Bolouri-Moghaddam et al. 2010). Glucose 6-phosphate dehydrogenase (G6PDH), catalyzing the first reaction in the oxidative pentose phosphate (OPP) pathway, has been postulated to affect the redox equilibrium of the chloroplast as well as the capacity to detoxify ROS (Debnam et al. 2004). Therefore, endogenous sugars feed the OPP and trigger (indirect) ROS scavenging. Moreover, sugar availability can enhance ascorbate biosynthesis (Linster et al. 2008; Bolouri-Moghaddam et al. 2010).

During plant defense reactions, invertase-related sugar signals seem to be very important (Roitsch et al. 2003; Bonfig et al. 2010). For instance, in tomato the LIN6 cell wall invertase is a pivotal enzyme for the integration of metabolic, hormonal and stress signals, regulated by a diurnal rhythm (Proels and Roitsch 2009). Hormone signals (e.g., cytokinines) often influence the expression of extracellular invertase genes (Lara et al. 2004), suggesting that such enzymes act as central modulators of assimilate partitioning, integrating sugar, stress, and hormone signals. Recent structural insights have led to a new point of view that not the invertases as such but rather the invertase/inhibitor complexes should be considered as central modulators (Hothorn et al. 2010). Perhaps, a similar function should be attributed to neutral invertase/PIP5K 9 complexes (Lou et al. 2007). Recent insights demonstrated that sugars can regulate the biosynthesis of auxins (LeClere et al. 2010), placing sugars (and sugar metabolizing enzymes and their complexes) at the heart of the regulatory processes that drive plant growth and development, both under normal conditions and under stress. In conclusion, numerous environmental and endogenous developmental and metabolic cues are integrated by sugar signals, operating in concert with plant-specific hormone signaling and stress-related pathways in a complex network (Bolouri-Moghaddam et al. 2010). It is not clear whether small fructans or RFOs can act as sugar signals too. Kawakami et al. (2008) hypothesized that fructans in transgenic rice could activate other stress signaling pathways leading to chilling tolerance. However, recent insights point to a more direct effect of fructans acting as true ROS scavengers (see next paragraph).

6 Sugars as Antioxidants: A New Concept

A stress-induced disturbance of the redox equilibrium in plant cells requires activation of antioxidant enzymes, primarily superoxide dismutase (SOD). However, a certain time is needed to accomplish additional SOD synthesis. Therefore, low molecular weight antioxidants (AsA, GSH) play a significant role at the initial stage of oxidative stress. In the last few years, also plant oligosaccharides have been proposed as emerging antioxidants in plants (Nishizawa et al. 2008; Foyer and Shigeoka 2011; Sinkevich et al. 2010; Stoyanova et al. 2011). They might play a role in scavenging hydroxyl and superoxide radicals.

6.1 RFOs and Galactinol as Antioxidants in *Arabidopsis*

In *in vitro* tests, the hydroxyl radical scavenging capacity of galactinol and raffinose was superior compared to those of typical antioxidants, such as AsA, GSH and citrulline (Nishizawa et al. 2008). The concentrations that reduce hydroxylation of salicylate by 50 % (ID_{50}; the concentration of a compound required to inhibit $^{\bullet}OH$ catalysed hydroxylation of salicylate (or terephthalate) by 50 % of the maximum yield observed in the absence of the compound) of galactinol, raffinose and stachyose were 3.1 ± 0.3, 2.9 ± 0.2 and 2.2 ± 0.1 mM, respectively (Nishizawa et al. 2008). For comparison, the concentrations of galactinol, raffinose and stachyose are 7.4, 0.74 and 3.6 mM in *Arabidopsis* seeds. This suggests that the initial intracellular levels of galactinol and stachyose might be in a good range to protect cellular components from oxidative damage (Nishizawa et al. 2008). Furthermore, raffinose concentrations in chloroplasts of stressed plants are estimated between 0.27 and 1.35 mM, comparable to the levels of AsA and GSH (Nishizawa et al. 2008). Stoyanova et al. (2011) reported an ID_{50} of 0.5152 mM for raffinose (hydroxyl radical) scavenging *in vitro*, suggesting that raffinose could act as a direct scavenger of hydroxyl radicals in chloroplasts too.

As already explained above, additional evidence for a role of galactinol and raffinose as antioxidants in plants was generated by paraquat treatments on plants overexpressing GolS (GolS1, GolS2, GolS4) and RafS. These transgenic plants showed more effective ROS scavenging capacity and oxidative stress tolerance compared to wild-type plants (Nishizawa et al. 2008). It can be speculated that RFOs might be operating as ROS scavengers in chloroplasts which is a major point of ROS production under different stress conditions (Foyer and Shigeoka 2011). The oxidized RFO radicals might be regenerated to RFOs by ascorbic acid (AsA) or other reducing antioxidants.

6.2 Disaccharides and Sugar Alcohols as Antioxidants

In vitro studies further demonstrated that the ID_{50} values of other sugars and sugar alcohols were even better than the ones obtained for raffinose and galactinol. Compared to mannitol, a widely recognized osmoprotectant, sucrose and especially inulin-type fructans show excellent antioxidant properties *in vitro* (Stoyanova et al. 2011). Some plant species such as sugarcane and sugar beet accumulate sucrose to extremely high levels (2 M) in their vacuoles. Therefore, sucrose might be a good candidate to act as a ROS scavenger in their vacuoles. Of course, such vacuolar antioxidant mechanisms occur in concert with the classic cytosolic antioxidant mechanisms (Van den Ende and Valluru 2009). So, at lower sucrose concentrations, sucrose might preferably act as a signal molecule, while it might become a ROS scavenger at high concentrations (Bolouri-Moghaddam et al. 2010).

Accordingly, transgenic potato plants carrying a yeast invertase gene (B33-inv plants), with decreased sucrose efflux and 20–30 % higher total sugar contents, showed enhanced cold tolerance and lower level of malondialdehyde (MDA; Sinkevich et al. 2010). MDA is an indicator of lipid peroxidation (LPO). The reduced MDA levels suggest that there is less ROS-mediated membrane damage in the transgenic potatoes, despite the higher levels of superoxide found in stressed B33-inv plants. These authors speculated that sugars act as primary antioxidants, and that the generated sugar radicals are reduced by AsA.

A group of sugar alcohols (such as mannitol, inositol, sorbitol) also possesses ROS scavenging capacities (Shen et al. 1997; Stoyanova et al. 2011). Mannitol protects thioredoxin, ferredoxin, GSH and the thiol-regulated enzyme phosphoribulokinase in *Nicotiana tabacum*. Genetically engineered tobacco plants with increased chloroplastic mannitol showed increased tolerance under paraquat treatments. In the same experimental setup, mannitol did not reduce $^{\bullet}OH$ radical production in the chloroplast, but it increased the capacity to scavenge these radicals protecting the cells against oxidative damage (Shen et al. 1997). Furthermore, mannitol accumulation had no harmful effects on these plants. This means that no sugar-mediated negative feedback on photosynthesis was observed, as is the case for metabolizable sugars such as glucose, fructose and sucrose (Bolouri-Moghaddam et al. 2010).

Trehalose also can act as a ROS scavenger *in vitro* (Stoyanova et al. 2011) and it was demonstrated that this sugar acts as a ROS scavenger *in vivo* in yeast (Nery et al. 2008). Exposing yeast to exogenous H_2O_2 leads to trehalose accumulation, which reduces the oxidant-induced modifications of proteins and the levels of lipid peroxidation. Transgenic rice plants accumulating increasing levels of trehalose showed increased tolerance to salt, drought and low-temperature stresses. Moreover, several transgenic lines exhibited sustained plant growth, less photo-oxidative damage and a more favorable mineral balance under stress (Garg et al. 2002).

6.3 *Fructans: A Role in Vacuolar Antioxidant Mechanisms?*

Fructans as water-soluble vacuolar oligo- and polysaccharides are probably good candidates to act as vacuolar ROS scavengers. Fructans can accumulate to a great extent in plant tissues (up to 20 % on a fresh weight basis; Van Laere and Van den Ende 2002). This kind of fructan levels cannot be solubilized *in vitro*, but apparently they can be kept in a solubilized (gelly-like) state in the vacuole, where they might interact profoundly with the inner side of the tonoplast. Under stress, when the redox equilibrium is disturbed, a spike in ROS occurs. The excess cytoplasmic H_2O_2, derived from ROS produced in chloroplasts or other cell compartments, can

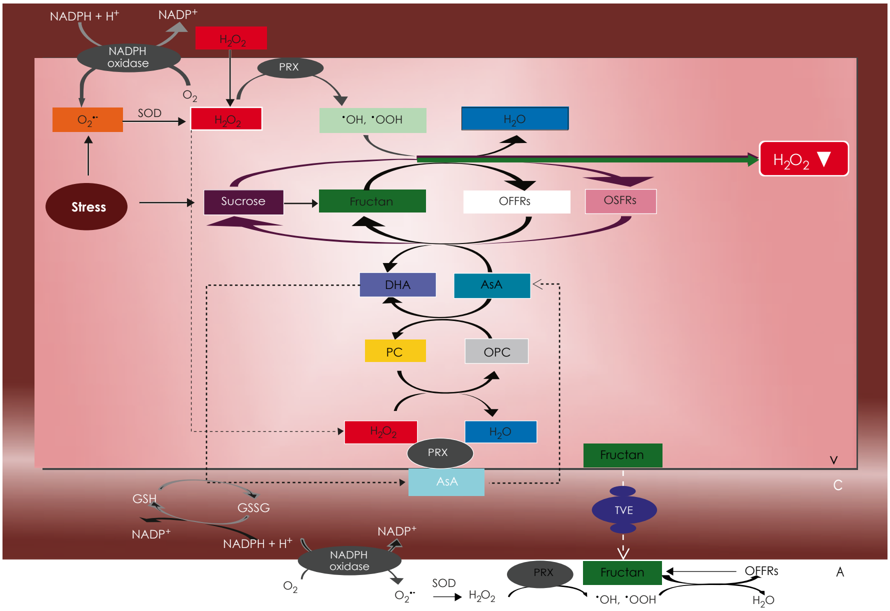

Fig. 13.2 Possible vacuolar scavenging mechanisms of fructans and sucrose in oxidative stress defense.
A apoplast, *AsA* ascorbate, *C* cytoplasm, *DHA* dehydroascorbate, *GSH* reduced glutathione, *GSSG* oxidized glutathione, H_2O_2 hydrogen peroxide, $O_2^{\bullet -}$ superoxide ion, $^{\bullet}OH$ hydroxyl radical, *OFFRs* oxidized fructan-free radicals, *OSFRs* oxidized sucrose-free radicals, *OPC* oxidized phenolic compounds, *PC* phenolic compounds, *PRX* peroxidase, *SOD* superoxide dismutase, *TVE* tonoplast vesicle-derived exocytosis, *V* vacuole

be directed to the vacuole (plant cell “detoxification factory” and “dump site”). H_2O_2 can diffuse through the tonoplast directly and/or through aquaporins. Another possible way to transmit the oxidative stress from the cytosol into the vacuole is through a putative vacuolar NADPH oxidase. Carter et al. (2004) reported this enzyme at the tonoplast but its localization was never confirmed by other studies (Fig. 13.2). This NADPH oxidase may be positioned in the neighbourhood of SOD and tonoplast-bound class III peroxidases (Fig. 13.1) catalyzing the reduction of H_2O_2. They use various substrates as electron donors, such as phenolic compounds, lignin precursors, auxin or secondary metabolites. However, as by-products of the so-called hydroxylic cycle of these peroxidase enzymes, the dangerous $^{\bullet}$OH and $^{\bullet}$OOH can be produced. The localization of fructans along the tonoplast make them ideally positioned to stabilize the tonoplast, but also to temporarily scavenge the aggressive $^{\bullet}$OH and $^{\bullet}$OOH radicals that are produced in the vicinity of these membranes (Fig. 13.1). The neutralization of these highly toxic radicals by the fructans or other vacuolar sugars/sugar-like compounds results into (less harmful) radicals (Fig. 13.1). It has been proposed that such sugar radicals could be recycled back into sugars with the help of phenolic compounds or anthocyanins with the use of AsA, GSH and cytosolic NADPH as final reductors (Fig. 13.2). For a long time, it was not clear whether AsA and GSH could cross the tonoplast. However, a recent report suggests that vacuolar accumulation of GSH is part of the oxidative stress response (Queval et al. 2011). In addition, AsA concentration in vacuoles was reported to increase four-fold under high light conditions that may cause oxidative stress (Zechmann et al. 2011). Additionally, oxidized sugar-free radicals may react with phenolic radicals (oxidized phenolic compounds), products of vacuolar peroxidases, to form complex sugar-phenol compounds. In resurrection species, such compounds considerably increase during drying (Moore et al. 2005). Notably, phenolic radicals may also combine to form polymers (Ferreres et al. 2011). Alternatively, they might be recycled, perhaps with assistance of vacuolar AsA and/or GSH (Fig. 13.2). Thus, vacuolar sugars or sugar-like compounds, present in the vicinity of the tonoplast and interacting with this membrane, might fulfill crucial roles in scavenging radicals and thus preventing lipid peroxidation by excess H_2O_2 produced under stress conditions. Perhaps phenolic compounds and fructans (or other vacuolar, sugar-like compounds) might operate in a synergistic way to scavenge excess vacuolar H_2O_2 (Bolouri-Moghaddam et al. 2010). The glucose that is produced by 1-SST during fructan biosynthesis may directly fuel, after retranslocation to the cytoplasm, the biosynthesis of classical antioxidants such as AsA (Bolouri-Moghaddam et al. 2010).

The reactions of fructan-accumulating transgenic plants (see above) fit well with the concept that these sugars can act directly as ROS scavengers. Notably, the dynamics of fructan concentrations in immature wheat kernels showed a close correlation with changes in concentrations of classical antioxidants such as AsA and GSH, suggesting a close cooperation of cytosolic and vacuolar antioxidant mechanisms (De Gara et al. 2003).

In conclusion, various vacuolar compounds and in particular sugars come into the picture as important new players in the defense against oxidative stress. Furthermore, many of these compounds are important food additives or are present in medical extracts. Therefore, the enzymes of sugar and phenol metabolism are interesting targets to improve crop yield, stress tolerance and to delay senescence in crop plants as well as to improve food quality (Bolouri-Moghaddam et al. 2010).

7 Opportunities for Crop Improvement through Sugar Metabolism

Today, mankind is challenged by increasing frequencies of Global Warming-associated abiotic stresses (e.g., drought, heat, and temperature extremes), decreases in arable land and rapidly growing populations with higher living standards. As a consequence, world crop yield needs to be doubled in the next 50 years (Ruan et al. 2010). This should be accomplished without destroying extra forests for arable land. Therefore, mankind needs stress-tolerant crops with higher yields per acre. A considerable part of world crops are so-called “reproductive crops”, relying on plant's reproductive structures such as grains, fruits, nuts and flowers. For example, in the USA 75 % of its harvested acreage is devoted to reproductive crops. The drastic and fast changes in climate, even for a short period of time, can greatly affect all crops but reproductive crops in particular. Drought and heat stresses during the time of flower development and pollination lead to irreversible damage, leading to fewer grains or fruits. Stresses occurring later in reproduction generally lead to smaller grains or fruits but this can often be reversed when the stress is relieved. However, in a case of fruit abortion the losses are permanent (Ruan et al. 2010).

Wheat is an economically-important crop. Worldwide, terminal drought is causing massive yield losses. Grain yield depends on carbon from two resources: flag leaf photosynthesis and remobilization of water soluble carbohydrates, mainly fructans, from the wheat stems (Yang and Zhang 2006). The production of new photosynthesis products may become limited under drought stress, due to decreases in leaf stomatal conductance and net CO_2 assimilation. Therefore, the contribution of stored carbohydrates may become the predominant source of transported materials (Plaut et al. 2004). Reserve pools have been estimated to contribute up to 10–12 % of the final wheat grain yield. This contribution further increases up to 40 % under drought and heat stress (Davidson and Chevalier 1992). The total amount of wheat stem fructans depends on the expression and activity of FTs (Xue et al. 2008). More importantly, however, efficient fructan mobilization depends on the activity of FEHs (Joudi et al. 2011,) Next to the focus on wheat stem fructans, also the dynamics of fructan pools in the reproductive organs (Ji et al. 2010) and in wheat kernels (Paradiso et al. 2006) deserve further attention for optimizing grain yield under terminal drought.

Besides drought, heat is one of the major types of abiotic stress experienced by most plants in the field. Heat and drought often come together but not always. Compared to drought, much less is known about plant responses under heat. However, among various cellular and metabolic responses, it appears that impairment of carbon metabolism and utilization are central factors causing abnormal development and yield losses under heat stress (Ruan et al. 2010).

Since carbohydrates constitute about 90 % of plant biomass, they form a crucial yield determinant. Sugar metabolic enzymes, particularly those acting on sucrose and starch, play major roles in regulating carbohydrate partitioning, plant development, and crop yield and quality (Ruan et al. 2010), especially under stress. For instance, tomato flowers are sensitive to heat stress. It was suggested that pollen development is strongly affected from heat stress than the female organs (Ruan et al. 2010). The observed decrease in pollen viability is caused by decreasing starch accumulation in developing pollen grains and low total soluble sugar in the anther wall (a similar scenario is observed in maize ovaries under water deficit). It was hypothesized that normal anther development depends on the activity of cell-wall invertases, which seems to be affected under heat stress, perhaps by increasing levels of cell wall invertase inhibitors (Frank et al. 2009). Therefore, downregulation of cell wall invertase inhibitors might be a keen strategy to increase crop yield under stress (Jin et al. 2009). Likewise, derepression of cell wall invertase activity appears to be a crucial event during the initial interactions with pathogens (Bonfig et al.

2010). By contrast, downregulation of invertases or an upregulation of invertase inhibitors proved to be a very efficient strategy to prevent cold-induced sweetening in potato tubers, in order to maintain the quality of chips and French fries, and to avoid browning and the formation of toxic acrylamides during the baking process (Bhaskar et al. 2010).

Cold stress is an important threat for the crops, especially during early growth stages and seed emergence (Zinn et al. 2010; Ohnishi et al. 2010). Rice, one of the major crops, is a good example of a species that is very sensitive to chilling. Expansion of rice cultivation into regions that experience periodic or sustained low temperatures, such as the Hokkaido region of Japan, has increased the risk of crop loss through chilling injury (Kawakami et al. 2008). Under low temperature conditions, rice suffers from leaf turgor losses and prolonged low temperatures result in leaf dehydration (Kawakami et al. 2008). It is a known fact that chilling-sensitive plants experience increased oxidative stress at temperatures not far above 0 °C (Valluru and Van den Ende 2008). Although chilling and frost tolerance are generally considered as complex multigenic treats (Hughes and Dunn 1996), it was rather spectacular and unexpected to see that chilling tolerance could be achieved in rice (Kawakami et al. 2008) by introducing a single gene encoding a 1-SST for fructan biosynthesis, counteracting oxidative stress (see above). However, introducing FTs in non-fructan accumulating crops may not always be that easy. For instance, introducing fructan synthesis in *Arabidopsis* proved to be difficult, only resulting in a minor accumulation of low DP fructans (Valluru and Van den Ende 2008). Perhaps, this can be explained by the high invertase and/or FEH activities in this species. It should be highlighted again that, besides the actual presence of a particular sugar as antioxidant and/or osmoprotectant, the exact subcellular location of such compounds is even more important for their functionality (Iftime et al. 2011).

In conclusion, the sensitivity of crops to harsh climates and soil conditions is a major limitation for worldwide food production. Worldwide, researchers look for new, desirable traits and their genes in extremophiles and resurrection plants, species that can survive extreme stress conditions (Amtmann 2009; Otto et al. 2009). Among many opportunities for crop improvement, the introduction of specific sugars (as antioxidants/osmoprotectants) and the modulation of key enzymes in sugar metabolism (or their inhibitors/partners) are very promising strategies to produce stress-tolerant crops with higher yield and quality.

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