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REVIEW ARTICLE

Plant fructans in stress environments: emerging concepts and future prospects

Ravi Valluru[1] and Wim Van den Ende[2,*]

[1] *Institute for Crop Production and Grassland Research, University of Hohenheim, D-70599 Stuttgart, Germany*

[2] *Laboratory for Molecular Plant Physiology, KU Leuven, Kasteelpark Arenberg 31, B-3001, Leuven, Belgium*

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Abstract

Plants are sessile and sensitive organisms known to possess various regulatory mechanisms for defending themselves under stress environments. Fructans are fructose-based polymers synthesized from sucrose by fructosyltransferases (FTs). They have been increasingly recognized as protective agents against abiotic stresses. Using model membranes, numerous *in vitro* studies have demonstrated that fructans can stabilize membranes by direct H-bonding to the phosphate and choline groups of membrane lipids, resulting in a reduced water outflow from the dry membranes. Inulin-type fructans are flexible random-coiled structures that can adopt many conformations, allowing them to insert deeply within the membranes. The devitrification temperature (T_g) can be adjusted by their varying molecular weights. In addition, above T_g their low crystallization rates ensure prolonged membrane protection. Supporting, *in vivo* studies with transgenic plants expressing FTs showed fructan accumulation and an associated improvement in freezing and/or chilling tolerance. The water-soluble nature of fructans may allow their rapid adaptation as cryoprotectants in order to give optimal membrane protection. One of the emerging concepts for delivering vacuolar fructans to the extracellular space for protecting the plasma membrane is vesicle-mediated, tonoplast-derived exocytosis. It should, however, be noted that natural stress tolerance is a very complex process that cannot be explained by the action of a single molecule or mechanism.

Key words: Abiotic stress, freezing tolerance, fructan, inulin, membrane stabilization.

Introduction

Plants cannot usually utilize all the carbon skeletons resulting from photosynthesis. Therefore, they store carbon skeletons as short or longer term reserve carbohydrates. Several pathways that link the generation, utilization, and storage of various storage carbohydrates dominate plant intracellular metabolism. Fructans are recognized as one of the principal stored forms of energy in 15% of higher plants (Hendry, 1993), as well as in a wide range of bacteria and fungi (Van Hijum *et al*., 2003; Martinez-Fleites *et al*., 2005). They are believed to be synthesized from sucrose in the central vacuole of plants (Frehner *et al*., 1984), although synthesis in prevacuolar vesicles can not be excluded (Kaeser, 1983). Different types of fructan molecules can be distinguished depending on the linkage type between the fructosyl residues and the position of the glucose residue (Lewis, 1993). Fructans with a terminal glucose residue include the β(2,1) type fructans (inulin, principally occurring in dicots), and the linear β(2,6) (levan) or branched type fructans (graminan) with both β(2,6) and β(2,1) linkages (as occurring in bacteria and monocots). Fructans with an internal glucose residue include the neo-inulin and neo-levan types (occurring in monocots such as *Lolium*, *Asparagus*, and *Allium*: Fujishima *et al*., 2005; Ueno *et al*., 2005).

In dicots, inulin-type fructans accumulate as long-term reserve carbohydrates in underground storage organs such as roots and tubers (Van den Ende and Van Laere, 2007). In grasses, graminan, levan, and neokestose-derived fructans mainly act as short-term storage compounds in stems, tiller bases, leaf sheaths, elongating leaf bases, and to a lesser extent in leaf blades and roots (Maleux and Van den Ende, 2007). However, fructans accumulating in perennial grasses can also be considered as longer term

* To whom correspondence should be addressed. E-mail: wim.vandenende@bio.kuleuven.be

reserve carbohydrates to survive the winter period (Yoshida *et al*., 1998). The predominant role for fructans is to bridge the temporal gaps between resource availability and demands. However, they can also fuel rapid regrowth in grasses (Morvan-Bertrand *et al*., 2001); regulate osmosis during flower opening (Le Roy *et al*., 2007*a*), and protect plants against cold and drought stress through membrane stabilization (Hincha *et al*., 2003). In addition to the plant world, inulin-type fructans have gained importance as functional food ingredients (Roberfroid, 2007) and have been shown to be effective against chronic inflammatory bowel disease (Leenen and Dieleman, 2007). Moreover, dietary fructans lead to an increase of the amine production in the intestine of animals preventing pasture-associated laminitis disease (Crawford *et al*., 2007).

Fructans are acid-labile, water soluble, and polydisperse polymers of fructose built upon a sucrose starter unit. The structure is unusual because the sugar rings are exterior to the polymeric chain of carbon and oxygen atoms (Fig. 1; Carpita *et al*., 1991; Zimeri and Kokini, 2003). The five-membered furanose rings are linked by a -O-CH_2- that make a much more flexible structure than the six-membered pyranose rings linked by an -O- in starch (Phelps, 1965). A loose helix with 4 or 5 fructosyl units per turn occurs in fructans, providing additional flexibility (John, 1991; French and Waterhouse, 1993; Vereyken *et al*., 2003). In levan, the furanose rings are part of the backbone, whereas inulin can be considered as a polyethylene oxide backbone with furanose rings attached to it. This configuration makes inulin-type fructans more flexible than levan-type fructans (Vereyken *et al*., 2003).

The synthesis of these structurally complex polymers is achieved via the co-operation of a suite of fructosyltransferases (FTs). According to the legendary SST/FFT model established exactly 40 years ago (Edelman and Jefford, 1968), sucrose:sucrose 1-fructosyltransferase (1-SST; EC 2.4.1.99) catalyses the production of the trisaccharide 1-kestose and glucose. Further elongation is achieved by fructan:fructan 1-fructosyltransferase (1-FFT; EC 2.4.1.100). Some species (*Echinops ritro*, *Viguiera discolor*) accumulate inulins with a higher degree of polymerization (DP) because they contain special high DP 1-FFTs (Van den Ende *et al*., 2005*b*, 2006).

In cereals, sucrose:fructan 6-fructosyltransferase (6-SFT) synthesizes the tetrasaccharide bifurcose (1&6-kestotetraose) by transferring a fructosyl unit from sucrose to 1-kestose (Duchateau *et al*., 1995). Further β(2,6) and β(2,1) linked fructosyl chain elongation to graminans (up to DP 20) is established by 6-SFT and fructan:fructan 1-fructosyltransferase (1-FFT), respectively (Jeong and Housley, 1992; Kawakami and Yoshida, 2005). Fructan:fructan 6G-fructosyltransferase (6G-FFT) is a key enzyme involved in the biosynthesis of neoseries fructans in *Lolium perenne* (Lasseur *et al*., 2006). Fructan

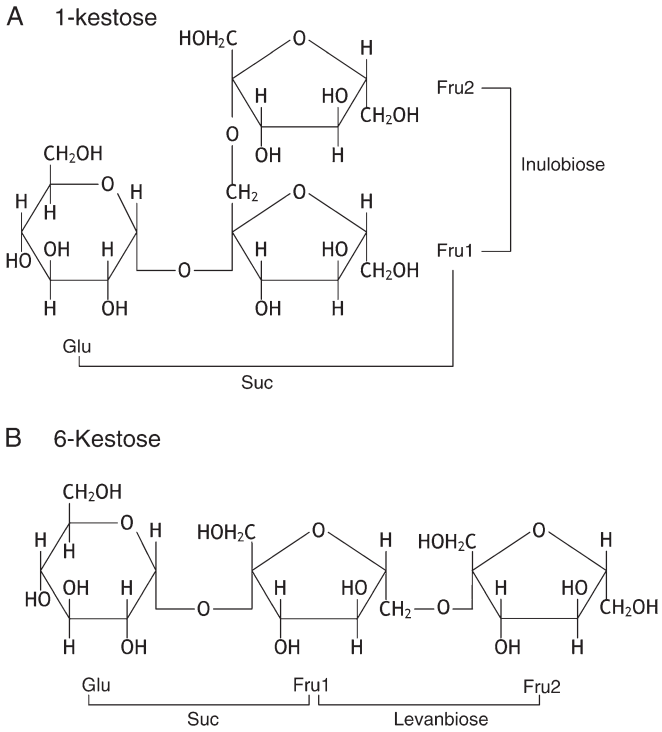

Fig. 1. The structure of 1-kestose (A), the smallest inulin, and 6-kestose (B), the smallest levan-type fructan.

breakdown in plants is accomplished by fructan exohydrolases (FEHs), releasing terminal fructosyl units using water as an acceptor. Different types of FEHs (1-FEH, 6-FEH, 6-KEH, and 6&1-FEH) have recently been described in fructan- and non-fructan plants (De Coninck *et al*., 2007). Recently, a third fructan 1-exohydrolase (1-FEHw3) has been purified from wheat (Van Riet *et al*., 2008). These FEH enzymes lack invertase activity.

The co-ordinated regulation between FT and FEH activities determine the fructan pool size in plants. To date, several FTs and FEHs have been identified and characterized at the biochemical and molecular level. In dicots, FTs and FEHs (1-SST and 1-FEH, respectively) are temporally separated (Van Laere and Van den Ende, 2002). By contrast, FEHs are co-expressed with FTs in monocots (Van den Ende *et al*., 2003). It is believed that FTs have evolved from vacuolar type invertases (VIs) while FEHs probably emerged from cell-wall invertases (CWIs). Both FEHs and CWIs might have evolved from a common ancestor, a β-fructosidase (Le Roy *et al*., 2008). Supporting this hypothesis, some of the FEH enzymes are active in the apoplast (Livingston and Henson, 1998; Van den Ende *et al*., 2005*a*). Within the CWI/FEH class of enzymes, the presence (CWIs) or absence (FEHs) of an 'Asp239' homologue defines the donor substrate specificity (Le Roy *et al*., 2007*b*). Together with the unexpected finding that FEHs occur in plants such as *Arabidopsis thaliana*, known as

a non-fructan accumulating plant, led to a new hypothesis that these FEHs are to be considered as 'catalytically defective invertases'. To date, two of six putative CWIs from Arabidopsis (AtcwINV3, 6) were proved to be defective invertases or FEHs (AtcwINV3=6-FEH; AtcwINV6=6&1-FEH) hydrolysing fructans but not sucrose (De Coninck *et al*., 2005). In addition, 'AtcwINV5' is predicted to be an FEH as well (Le Roy *et al*., 2007*b*). These FEHs might be involved in signalling and defence responses or may fulfil novel, non-catalytic roles in plants (Van den Ende *et al*., 2004; Le Roy *et al*., 2007*b*).

Fructans are synthesized in the plant vacuole. In addition, an increasing body of evidence shows that fructans can also be present in the apoplast, phloem, and xylem tissues (Vieira and Figueiredo-Ribeiro, 1993; Livingston and Henson, 1998; Wang and Nobel, 1998; Ernst and Pfenning, 2000; Van den Ende *et al*., 2000, 2005*a*). A recent study demonstrated the long-distance transport of lower DP fructans and the accumulation in tubers of transgenic potato (Zuther *et al*., 2004). The presence of fructans and FEHs in the apoplast strongly suggested that fructans and their degradation products are involved in abiotic stress tolerance by directly interacting with membrane lipids (Van den Ende *et al*., 2005*a*). In this review we focus on the possible pathways mediating abiotic stress tolerance considering different physiological scenarios.

Evolution of the fructan syndrome: a link to abiotic stresses?

Environmental factors primarily govern the geographical and ecological breadth of many economically important species. Strikingly, fructan-accumulating species are more prominent in cold and dry environments and absent in tropical and aquatic environments (Hendry, 1993). The capability to synthesize fructans (the 'fructan syndrome') was gained by the development of an array of FTs from different types of ancestral vacuolar invertases (Ritsema *et al*., 2006). Essentially, the FT enzymes developed the capacity to use sucrose or fructans instead of water as an acceptor substrate. It is tempting to speculate that the lower cellular water content (and higher cellular sucrose content) by desiccation (or subzero temperatures, since desiccation forms an integral part of the freezing process) might have been the driving force during this evolutionary process.

Fructan content and its metabolism have been shown to be closely related to frost and drought tolerance (Trunova, 1965; Pontis, 1989; Tognetti *et al*., 1990; Yukawa and Watanabe, 1991; Santoani *et al*., 1993; Yoshida *et al*., 1998; De Roover *et al*., 2000). Interestingly, a transition from aquatic to dry terrestrial environments reported increased fructan levels in an amphibious, freshwater plant *Littorella uniflora* (Robe and Griffiths, 2000). One general conclusion can be drawn from all experimental work described so far: accumulation of low DP fructan (either by FT activity or by partial breakdown of earlier formed fructans, see below) is always observed under abiotic stress conditions.

Overall, FT gene expression seems to be mainly (and perhaps directly) controlled by the level of sucrose (Van den Ende and Van Laere, 2007; Gallagher *et al*., 2007) rather than directly by cold or drought. Mild drought and cold stress conditions are known to arrest growth but not photosynthesis, leading to excess spillover of sucrose concentration acting both as an inducer of fructan biosynthetic genes and as a substrate for the sucrose splitting FTs (De Roover *et al*., 2000; Maleux and Van den Ende, 2007). However, a direct effect of cold and/or drought on FT expression cannot be excluded (Kawakami and Yoshida, 2002). Variation in wheat stem WSC (water-soluble carbohydrate: fructan, sucrose, and hexoses) among wheat genotypes is one of the genetic factors influencing grain weight and yield under water-limited environments. Xue *et al*. (2008) found that the mRNA levels of 1-SST and 6-SFT in the wheat stem were positively correlated with stem total WSC and fructan concentrations, demonstrating that fructan biosynthetic pathways are intimately connected to abiotic stresses. Hisano *et al*. (2008) proposed a complex, co-ordinated regulation of several FTs under low temperature.

Membrane stability under abiotic stresses

Physical consequences of abiotic stress

Low temperature, drought, and salinity stresses represent the major limiting abiotic stress factors for agricultural productivity. Exposure to these stresses causes various perturbations in membrane structures, including membrane fusions and phase transitions. The plasma membrane, the most exposed cell organelle, is thought to be the major target for stress damage due to changes in composition and structures of water and lipid bodies (Levitt, 1980). Water molecules play a vital role in maintaining the structural integrity and proper functioning of lipid bilayers by making direct hydrogen bonding bridges between the lipid head groups (Milhaud, 2004; Beck *et al*., 2007). In addition, water is needed for the interaction between (and activity of) soluble enzymes at the membrane interphase (Martini and Disalvo, 2007).

Exposure to subzero temperatures results in extracellular freezing of tissues and reduced water potentials promoting migration of cellular water to extracellular ice masses (Heino and Palva, 2003). During slow extracellular freezing, water becomes incorporated in large extracellular ice masses causing osmotic cell contraction to a volume consistent with the Boyle–van't Hoff relationship (Guy,

2003). The removal of water molecules between lipid bodies creates a close approach among lipid headgroups. This increased packing, in turn, leads to increased opportunities for van der Waals interactions among the hydrocarbon chains and increases the gel to liquid-crystalline phase transition temperature (T_m) (Ricker *et al*., 2003). The increased T_m results in the transition from a highly fluid, liquid-crystalline phase to a more rigid gel-phase, in which lipids are closely packed, highly ordered, and can render the membrane more permeable and prone to rupture (Cyril *et al*., 2002). The disorder and disassembly within a lipid bilayer could contribute to the fluidity of the membrane and membrane leakage (Verslues *et al*., 2006; Beck *et al*., 2007), which could be demonstrated by using fluorescence markers (carboxyfluorescence, CF) and lipid vesicles (Hincha and Hagemann, 2004).

Membrane protection by simple sugars

Severe abiotic stresses cause detrimental changes in cellular compounds. Sugars can be regarded as one of the metabolites that can prevent these detrimental changes. Their role in abiotic stress tolerance and their direct interaction with lipid bilayers have been substantiated (Hincha *et al*., 2006). Long-term stress conditions lead to higher soluble sugar concentrations and lower amounts of starch (Silva and Arrabaca, 2004). Both mono- and disaccharides interact with lipid membranes and are effective against abiotic stresses (Ohtake *et al*., 2006). Sugars may fulfil two distinct roles in membrane protection under drought: (i) reducing the phase transition temperature (T_m); and (ii) forming an amorphous carbohydrate glass with high melting temperature T_g (Oliver *et al*., 2002). These mechanisms are not mutually exclusive and may operate in parallel to obtain an efficient membrane protection in the dry state (Crowe, 2002; Cordone *et al*., 2007). According to the (most widely accepted) water-replacement hypothesis, sugars can substitute water during drying. Sugars inhibit the fusion of biological membranes by interacting directly with the polar headgroups of membrane phospholipids (through hydrogen bonds) essentially replacing the water molecules upon dehydration. In addition, sugars inhibit the fluid to gel membrane phase transition at low hydration, thereby stabilizing the native structures of the lipid bilayers (Ohtake *et al*., 2006). A recent study demonstrated that a maximal protective effect is reached at a ratio of about 1.5 sugar rings (glucose or sucrose) per lipid molecule (Lenne *et al*., 2007). This study supports previous works on trehalose (Crowe and Crowe, 1988; Nakagaki *et al*., 1992). Interestingly, an equal sugar/lipid ratio was also found effective in reducing the T_m (Cacela and Hincha, 2006*a*).

'Sugar vitrification' is a common feature explaining the formation of a solid, amorphous glass that prevents membrane fusion. The formation of a glassy state does not imply hydrogen bonding, as shown by measurements on dextran (Allison *et al*., 1999). The magnitude of sugar vitrification depends on the temperature at which glass devitrifies (T_g), which itself depends on the molecular weight of the sugar and on the water content (Slade and Levine, 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. However, the presence of certain sugar components in specific regions of the plant may not be sufficient for whole plant survival under stress conditions. For example, glucose can interact directly with lipid headgroups, but has a low T_g value and devitrifies at relatively low temperatures, not preventing membrane fusion during dehydration (Oliver *et al*., 2002). This indicates that alternate mechanisms and physical parameters may also determine the ability of different sugars in membrane protection against fusion during drying (Hincha *et al*., 2007). Moreover, the protective effects of carbohydrates during the drying of liposomes are based on the interaction between the sugar and the lipid and the glass-forming properties of the carbohydrates. Direct interaction is pivotal to prevent leakage through the bilayers, whereas the formation of stable glass prevents fusion.

Membrane protection by polysaccharides

Polysaccharides have paramount importance in sugar vitrification mechanisms. In the working lines of sugar vitrification, increasing devitrification temperature (T_g) inhibits liposome fusion in the dry system and provides superior protection at elevated temperatures (Hincha *et al*., 2003). Membrane protection by sugars has been shown to be closely associated with their ability to vitrify during drying, depending on, among other factors, the molecular weight of the sugars which consistently follows the Fox-Flory equation (Hinrichs *et al*., 2001). Extensive studies demonstrate that T_g increases with increasing molecular weight (Oliver *et al*., 2002) and DP of sugars (Slade and Levine, 1991). The relative relationship between T_g and molecular weight is due to a decrease in chain ends, which largely contributes to the free volume (Slade and Levine, 1991). Supporting this, previous studies have demonstrated the protective effects of many oligosaccharides, such as raffinose family oligosaccharides (RFO) (Buitink *et al*., 2000; Hincha *et al*., 2003), fructo-oligosaccharides (Hinrichs *et al*., 2001; Hincha *et al*., 2002), malto-oligosaccharides (Orford *et al*., 1989), and muco-polysaccharides (hyaluronic acid; Ionov *et al*., 2004).

Polysaccharides have been shown to possess higher T_g values than simple sugars (Oliver *et al*., 2002). In a study of inulin glasses for the stabilization of therapeutic

proteins, inulins have been reported to have a T_g value in the range of 102–154 °C (Hinrichs *et al*., 2001). Similarly, dextrans and hydroxyethyl starch (HES) seem to have comparably high T_g values (>100 °C) (Crowe *et al*., 1996, 1997; Koster *et al*., 2000). By contrast, simple sugars such as glucose (21–39 °C) (Hinrichs *et al*., 2001) and sucrose (67 °C) (Roos, 1995) show much lower T_g values.

Despite their higher T_g values, dextrans and HES are unable to depress the dry membrane T_m. Indeed, vesicle leaking experiments in the presence of these polymers in the dry state (Koster *et al*., 2000) confirmed their inability to interact directly with membrane headgroups (Crowe *et al*., 1997), because their larger size sterically prevents interaction with lipids (Wolfe and Bryant, 1999). These polymers thus have little direct effect on the bilayers and T_m, although they could show indirect effects by changing the overall water chemical potential, which slightly increases T_m (Koster *et al*., 2000).

Membrane stabilization by fructan

Special properties in membrane stabilization

Remarkably, only one class of polysaccharides, fructans, is able to interact with lipid headgroups in a similar way to disaccharides and raffinose-family oligosaccharides (RFOs). In the dry state, fructans can depress T_m and stabilize lipid bilayers (Hincha *et al*., 2000). Fructans show much more flexible structures compared with other polysaccharides such as dextrans and HES. Closer examinations of the fructan–lipid interaction revealed that they behave more like low molecular mass sugars, being capable of inserting a part of the polysaccharide body between the lipid headgroups. The addition of fructans resulted in a substantial reduction of water outflow from dry membranes (Demel *et al*., 1998; Hincha *et al*., 2000, 2007; Vereyken *et al*., 2003). In particular, it was suggested that, by binding lipid molecules more tightly, the glass formed by fructans could reduce molecular motions, leading to its bio-protective effects. These remarkable properties were explained by their variable molecular weight (Hinrichs *et al*., 2001) and their special polyethylene oxide backbone (French and Waterhouse, 1993; Vereyken *et al*., 2003).

Fructans are architecturally designed with three direct single bond linkages allowing rotations that impart a much greater flexibility than starch having only two-bond linkages (French and Waterhouse, 1993). In general, the torsion angles (φ, ψ, ω), the orientation of the primary alcohol groups, and puckering of the sugar rings impart differences in conformation and flexibility among polysaccharides. The torsion angle φ describes the bond from a reducing carbon to linkage oxygen, and the angle ψ describes rotation about the bond that links the linkage oxygen with a carbon of an adjacent residue. The ω angle explains the orientation of primary alcohol groups. However, in fructofuranose linkages, the angle ω also acts as a linkage torsion angle (French and Waterhouse, 1993). The central bond of inulobiose (C1f1-O1f2) usually takes *trans* conformation and the rotations between the C2f2-O1f2 and C2f1-C1f1 (Fig. 2) bonds are anticipated to be more flexible. The presence of the furanose ring exhibiting different forms enhances the flexibility at linkage bonds and at side groups. Thus, fructans provide the flexibility and capacity to replace water molecules. For example, a single raffinose molecule has a volume equal to ~30 water molecules, suggesting that a raffinose molecule could replace 30 water molecules compared to trehalose and sucrose (~18) (Carpita *et al*., 1979). Comparatively, 1-kestose has a volume equal to about ~21 water molecules. While it has the same φ torsion angle (O5g-C1g-O1g-C2f1) as raffinose, it differs in the ψ torsion angle (C1g-O1g-C2f-O5f1) by 80° (French and Waterhouse, 1993). A recent study demonstrated an increased protection with chain length for inulins compared with other malto- and manno-oligosaccharides (Cacela and Hincha, 2006*b*). Longer chain length inulins are less soluble, and they have the ability to form 'inulin-microcrystals' when sheared in water. Moreover, fructans have exceptional steric hindrances due to greater combinations of the driven torsion angles allowing greater variations in fructan ring confirmations and flexibility (Waterhouse *et al*., 1991). Recent studies suggest that glucan oligosaccharides that contain three dihedral angles (1,6 linkages) exhibit more flexibility than oligosaccharides having two dihedral angles (1,4 linkages) (Kony *et al*., 2004; Lee *et al*., 2004).

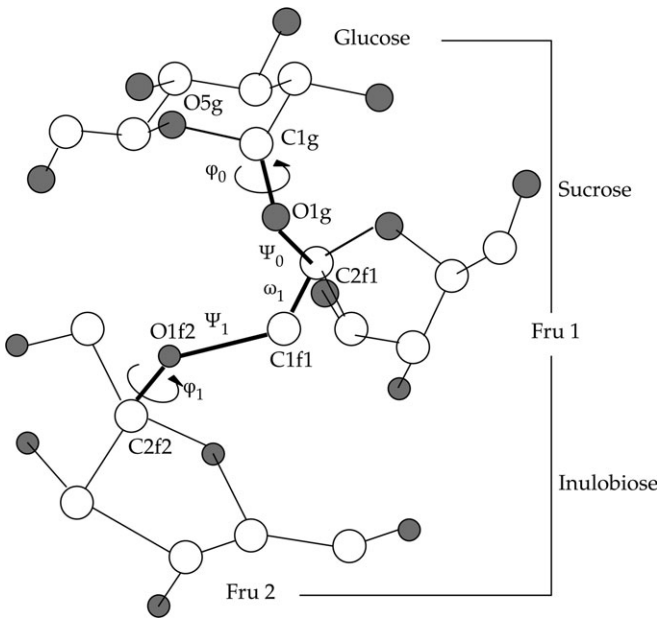

Fig. 2. Simple illustration of the torsion angles in 1-kestose.

A recent study built the most favourable conformations for inulin and levan-type fructans (Vereyken *et al*., 2003). They found a torsion angle ω at 180° for levanbiose (Fru 2,6 Fru); and 60° and 180° conformations for inulobiose (Fru 2,1 Fru). Based on these data, they built molecular dynamics (MD) simulations for DP 10 fructans. Levan of DP 10 showed 5 units per two turns (Fig. 3A), in which the first and last hydrogen bonds were estimated to occur at lower percentages, providing more flexibility (Vereyken *et al*., 2003). Interestingly, inulins with many conformations could not show any hydrogen bonds at both conformations, suggesting even more flexibility than levans (Fig. 3B, C). Based on these conformational data, the greater variability in torsion angles in inulin-type fructans compared with levan-type fructans explains why inulin-type fructans show a more profound interaction with membranes, making a direct hydrogen bond with the phosphate groups and even with the C=O groups (Fig. 2; Hincha *et al*., 2000; Vereyken *et al*., 2003; Cacela and Hincha, 2006*b*). These flexible structural arrangements depress T_m by more than 20 °C (Crowe *et al*., 1996; Hincha *et al*., 2000), suggesting that size–related effects of steric hindrance are exceptional for the observed effects of polymeric fructans (Hincha *et al*., 2002). They interact with lipid headgroups, which makes them immobile but allow mobilization and partition of acyl chains (Vereyken *et al*., 2003). Indeed, fructan vitrification during dehydration (Vereyken *et al*., 2003), which form a solid-like glassy structure (Hinrichs *et al*., 2001) could immobilize lipid headgroups and this was well correlated with a lower phase transition temperature. The presence of fructan modifies the tetrahedral hydrogen bond network and the water dynamics. An earlier study suggested that inulin has a water-binding capacity of about 2:1 (Silva, 1996). In addition, fructans could show a lower crystallization rate above the T_g providing prolonged protection of membranes (Hinrichs *et al*., 2001).

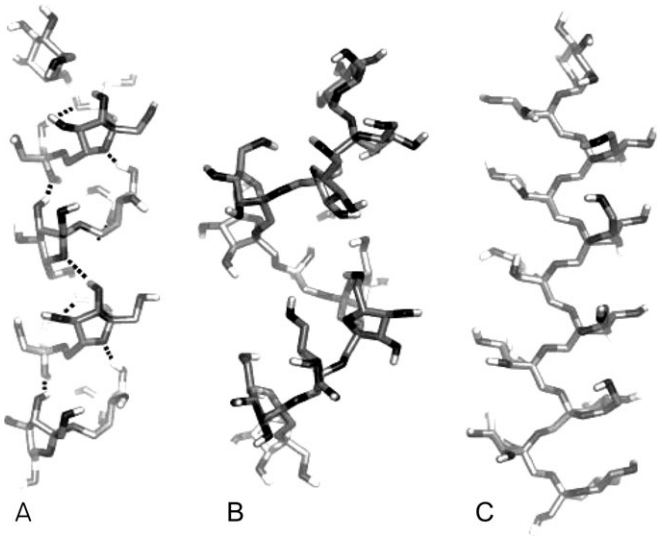

Fig. 3. MD simulations of fructan conformations. The preferred conformation of DP10 levan (A), a right handed helix with five units per two turns. Hydrogen bonds are indicated by dashed lines. Two favoured conformations of DP10 inulin, a right-handed 6-fold helical structure (B), with all ω dihedral angles at 60°, and a zigzag structure (C), with all ω dihedral angles at 180°. Reprinted with kind permission of Vereyken *et al*. and The Biophysical Society.

Stress tolerance in plants: high DP versus low DP fructans

As described above, high DP fructans are more efficient in stabilizing biological membranes under stress. Moreover, they are particularly interesting for specific food and non-food applications (Franck and De Leenheer, 2002; Van den Ende *et al*., 2005*b*). Most Asteracean species (e.g. *Cichorium* and *Helianthus*) are low DP inulin accumulators (mean DP~10, maximal DP~70) while only a few species (*Viguiera discolor* and *Echinops ritro*) accumulate high DP inulins (mean DP~30, maximal DP~300). Strikingly, *Echinops* and *Viguiera* are very drought-tolerant species (Van den Ende *et al*., 2005*b*, 2006). Interestingly, and similar to what is observed among dicot species, some monocot species also developed the capacity to synthesize high DP levan-type fructans (Maleux and Van den Ende, 2007). Some varieties from *Dactylis* and *Lolium* are able to survive severe droughts in the Mediterranean regions, and this was found to be correlated with their ability to accumulate high DP fructans (Volaire *et al*., 1998). Similarly, it was reported that cold-tolerant cereals show a tendency to accumulate higher DP fructans (Suzuki and Nass, 1988) but this was different in other cereals (Livingston *et al*., 2007). Taken together, it can be hypothesized that a mixture of both high DP and lower DP fructans (formed after partial hydrolysis by stress-induced FEHs) might provide superior membrane protection. The low DP fructans could provide the necessary sugar–lipid interaction (and decrease in T_m), while the high DP fructans could provide the high T_g (Crowe *et al*., 1997). Such synergism of protective compounds has also been reported using mixtures of trehalose and borate (Miller *et al*., 1998).

FEH activity on fructans generates low DP sugars, sucrose and hexoses

Cold stress can induce FEHs for degrading higher DP fructans into lower DP fructans and fructose. This depolymerization process has been extensively studied in chicory (Van den Ende and Van Laere, 2007, and references therein). Both fructan contents and FEH activities have also been measured in wheat (Yukawa *et al*., 1994, 1995; Yoshida *et al*., 1998; Van den Ende *et al*., 2003). Both fructans and FEHs were found in the apoplast (Livingston and Henson, 1998; Van den Ende *et al*., 2005*a*), leading to the hypothesis that apoplastic

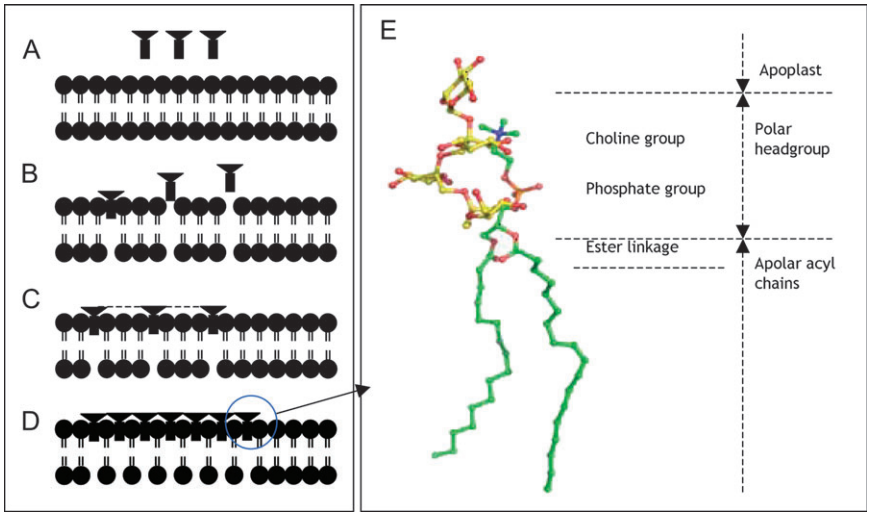

Fig. 4. Interaction between fructans and membrane lipids. In the initial stages of abiotic stresses, apoplastic fructans (A) move towards membranes. The close approach of fructans to the lipid headgroups allows insertion of a part of the fructan body (B, C) into the lipid headgroup through H-bonds. The deeply inserted fructan body may form a sheet-like structure (D) and thus prevent membrane leakage and preserve membrane stability. 1,1-nystose inserted between the headgroup of phosphatidylcholine membrane lipids (phosphate, orange; carbon, green; nitrogen, blue; oxygen, red) (E). Coordinates of 1,1 nystose and the membrane lipids were calculated using molecular dynamics (Timmermans *et al*., 1993; Heller *et al*., 1993). The picture was drawn with Pymol (Delano, 2003).

fructans could be trimmed by these FEHs, and thus generate a mixture of oligofructans, sucrose, and fructose that might be optimal for stabilization of the plasma membrane. Supporting this, it was calculated that the hexose sugars generated from fructan hydrolysis in oat could lower the water freezing point by a degree (Pollock *et al*., 1988; Livingston and Henson, 1998). Although this reduction in freezing point temperature is relatively low, solute concentrations in reducing freezing point seem to operate in a colligate manner in wheat (Johanssen, 1970). In addition, the uneven distribution of carbohydrates in the apoplast (Canny, 1995), only dissolving into a very small layer of water, may locally result in very high sugar concentrations. In this way ice adhesions might be prevented in the extracellular environment (Pearce and Ashworth, 1992).

FEH regulation

Persistent drought stress may lead to fructan degradation in cereals (Yang *et al*., 2004; Clark *et al*., 2004). Some dicot FEHs (Michiels *et al*., 2004; Itaya *et al*., 2007) are directly regulated by cold at the transcriptional level and by sucrose at the protein level (Verhaest *et al*., 2007). Interestingly, some monocot FEHs seem to be post-translationally regulated (Lothier *et al*., 2007). Taken together, these studies suggest a complex regulation of fructan metabolic genes by abiotic stresses and overall sugar status of the plant. Since sugars and hormonal signalling are intimately connected (Leon and Sheen, 2003), the hormonal balance most likely plays a role as well. Recently, ABA was reported to be particularly important for FEH induction in wheat (Ruuska *et al*., 2008).

Transgenic approaches to increase stress tolerance

Transgenic approaches offer powerful means to gain valuable information toward a better understanding of the mechanisms that govern stress tolerance. They also open up new opportunities to improve tolerance to stresses by incorporating a gene involved in stress protection from any source into agriculturally important crop plants, such as rice and wheat. Indeed, recent investigations convincingly demonstrated that introduction of lettuce 1-SST in tobacco (Li *et al*., 2007) and wheat 1-SST in rice (Kawakami *et al*., 2008) effectively contribute to fructan accumulation and freezing or chilling tolerance, respectively. Rice is a chilling-sensitive plant and wheat is a cold-acclimating and freezing-tolerant plant. Cold stress damage above 0 °C in a chilling-sensitive plant is thought to be caused, for example, by the formation of reactive oxygen species (ROS). Interestingly, recent data strongly suggest that raffinose and galactinol scavenge hydroxyl radicals as a novel function to protect plant cells from oxidative damage caused by chilling (Nishizawa *et al*., 2008). Moreover, transgenic tobacco plants carrying lettuce 1-SST and accumulating 1-kestose produce less malondialdehyde, an end-product of lipid peroxidation (Li *et al*., 2007).

All together, these transgenic experiments demonstrated for the first time that there is a real causal relationship (not a simple correlation) between fructan (or raffinose) accumulation and abiotic stress tolerance. Although earlier experiments supported similar conclusions, it should be

noticed that plants expressing bacterial levansucrases (Pilon-Smits *et al*., 1995, 1999; Konstantinova *et al*., 2002; Parvanova *et al*., 2004; Park *et al*., 1999) failed to direct the enzymes to the plant vacuole. Pleiotropic (toxic) effects were sometimes observed (reviewed in Cairns *et al*., 2003). No pleiotropic effects were observed in transgenic perennial ryegrass plants expressing wheat fructosyltransferase genes. These plants accumulated increased amounts of fructan and acquired increased tolerance to freezing (Hisano *et al*., 2004), but their endogenous fructan metabolism remained a point of criticism.

Although the introduction of wheat FT genes in rice now demonstrated that it is possible to change a non-fructan-accumulating plant into a more stress-tolerant fructan-accumulating plant, it is important to note that this technique might not be universally applicable. Indeed, efforts to introduce fructan metabolism in the model plant *Arabidopsis thaliana* only resulted in a minor accumulation of low DP fructans (A Heyer, personal communication), indicating that (i) Arabidopsis contains much more invertases and/or FEHs preventing fructan accumulation; and (ii) Arabidopsis might not possess the correct type of vacuole or prevacuolar vesicles allowing fructan accumulation. The ongoing debate on the number of vacuole types in Arabidopsis might be an important consideration in this respect (Rogers, 2008).

How do fructans reach the plasma membrane?

Although significant progress has been made in the fructan area, one intriguing question still remains unanswered: how do vacuolar fructans reach the plasma membrane? This is particularly important in order to protect the plasma membrane and its integral components. Since FTs are never present in the extracellular space (Van den Ende *et al*., 2005a), it is reasonable to assume that fructans must be transported from the zone of synthesis (vacuole) to the site of action (apoplast), where they could stabilize membranes under stress conditions. Recently, vesicle-mediated mechanisms are found to be highly important in many plant physiological processes, including stress responses (Mazal *et al*., 2004). Endocytotic traffic of sucrose has been demonstrated from the plasma membrane to the vacuole (Etxeberria *et al*., 2005). It can be speculated that a reverse, exocytotic pathway might exist in plants to carry vacuolar substances (such as fructans and simple sugars) to the extracellular spaces. However, further research is needed to generate hard evidence for this hypothetical fructan transport pathway.

Conclusions and perspectives

Fructans are fructose-based polymers synthesized from sucrose by FTs and hydrolysed by FEHs. In addition to their well-established localization to the vacuole, the exclusive appearance of apoplastic fructans and FEH activities strongly supports an adaptive role for fructans in mediating stress tolerance. Recent structural simulations on fructans have uncovered crucial and flexible conformations that might allow fructan interaction with membrane lipids. Indeed, inulin-type fructans seem to be arranged in random-coil structures with many conformations compared with levan-type fructans, which appeared to be arranged in helix-structures. Moreover, fructans can overcome size-related steric hindrances providing even more flexibility than other oligosaccharides. Such flexible structural arrangements might allow fructans to maintain direct H-bonding with the phosphate groups of lipids thereby reducing water leakage and protecting the structural integrity of membranes. The presence of apoplastic FEHs could support the partial hydrolysis of fructans generating an optimal mixture of higher and lower DP fructans, sucrose and hexoses, providing superior membrane protection (by influencing T_m and T_g) and/or establishing freezing point depression.

Many important gaps, however, remain to be filled in our understanding of fructan regulation in stress physiology, such as deciphering the exact roles of fructans in tonoplast and plasma membrane stabilization under stress, investigating whether fructans can accumulate in (prevacuolar) vesicles, and unravelling the precise mechanisms and regulation of vesicle-mediated tonoplast-derived fructan transport to the extracellular space.

These future perspectives, combined with plant biotechnology, will likely contribute to the introduction of the fructan trait in the edible parts of rice and other world crops. This would not only make rice more resistant to abiotic stresses but would also ensure the incorporation of healthy fructans into the diet of millions of people, in this way contributing both to our future needs on food quantity and quality.

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