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9 The relationship of fructan to abiotic stress tolerance in plants

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Abstract

Fructan has long been recognized as a crucial component of drought and freeze protection in plants but the relationship is controversial and many studies have shown contradictory correlations between fructan and resistance to stress. Much of the early research considered how hexose sugars from fructan hydrolysis affected the chemical potential of water. As model membrane systems became available, investigations to study the effect of fructan on liposomes were initiated. These studies indicated that a

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direct interaction between membranes and fructan was possible. This new area of research began to move fructan and its association with stress beyond mere correlation by confirming that fructan has the capacity to stabilize membranes during drying by inserting at least part of the polysaccharide into the lipid headgroup region of the membrane. This helps prevent leakage when water is removed from the system either during freezing or drought. In addition to evidence from studies with model membranes, when plants were transformed with genes encoding enzymes that enable them to synthesize fructan, a concomitant increase in drought and or freezing tolerance was confirmed. While exact mechanisms are still open for consideration it has become clear that besides the possibility of an indirect effect of supplying tissues with hexose sugars when the need arises, fructan may have a direct protective effect in plants that can be demonstrated by both model systems and genetic transformation. These studies may help breeders as they attempt to combine favorable genes into agronomically acceptable cultivars and extend their cultivation into regions where they currently cannot be grown.

1. Introduction

1.1. History of research relating fructan to abiotic stress

Research on fructan began in the early 1800's with the discovery of inulin in roots of *Inula helenium* by Rose [1]. Throughout the 1800's and until the early 1900's most fructan research involved experimentation with various extraction and quantitation methods that were used to identify plants that accumulate fructan [see review by Archibold, 2]. Pollock [3] reviewed discrepancies associated with various early extraction and quantitation methods. For example, the concentration of ethanol used during extraction was found to have a dramatic effect on the size classes of fructan extracted from various plant species [4,5] and may have been a factor in conflicting results from earlier research. Taking into account analytical problems with earlier studies, Hendry [6] reported fructan to be present in 10 plant families worldwide and most prominently in the Gramineae with 1200 species accumulating fructan. Meier and Reid [1] reported that 11 families of dicots and 6 families of monocots accumulated significant quantities of fructan.

In an analysis of carbohydrates (including fructan) from leaves of 185 genotypes of Gramineae, Chatterton et al. [7] reported a range of zero percent fructan up to 45% of dry weight. They also reported that fructan accumulated only in cool season grasses and not in warm season species. Some studies reported that fructan accounts for as much as 80% of the dry weight during environmental conditions that favored accumulation [1,8,9]. Meier and Reid [1] report concentrations as high as 90%.

Despite a wide range of reported concentrations, research clearly indicated that fructan accumulated in various tissues [10-12] during periods when light levels promoted carbon fixation while lower temperatures reduced growth [2,13]. Edelman and Jefford [8] proposed that fructan accumulated in the vacuole which provided a sink that would allow photosynthesis to continue. This compartmentation in the vacuole was confirmed in barley [14] and provided an explanation for the large concentrations of fructan in some species under conditions where low temperatures reduced growth rates while light levels promoted continued photosynthesis [see reviews 11,15,17].

The accumulation of fructan during periods of reduced growth, generally coincided with an increase in freezing tolerance of numerous species [13,18-20]. The finding that fructan content increases during cold acclimation prompted studies to explain how fructan could be involved in protection from stress during freezing as well as drought, since desiccation was shown to be an integral part of the freezing process [21-23]. With regard to abiotic stress, advantages of fructan accumulation over that of starch were listed as: 1) the high solubility of fructan in water, 2) the resistance to membrane-damaging crystallization at subzero temperatures and 3) the lack of sensitivity to cold of the synthesis pathway [19,24].

2. Fructan as an indirect source of hexose sugars

2.1. Protection from abiotic stress by sugars

The importance of simple sugars in the protection of plants from freezing injury [25-28] led to the speculation that fructan may act as a carbohydrate reserve that could supply hexose sugars to protect tissues in a colligative manner [20,29]. Colligative properties of ideal solutions, depend only on the number of solute particles in solution. One such property is freezing point depression. Johanssen [30] followed the freezing point of wheat at varying solute concentrations and found that freezing points were lowered on a "purely colligative basis". However, it has been observed that even if most of the fructan in oat was hydrolyzed, the increased hexose sugars would only lower the freezing point of water by a fraction of a degree [31,32]. Levitt [21] reports the highest recorded vegetative plant cell-sap concentration would lower the freezing point by only 4°C. This prompted some authors to question whether a relationship between sugars and protection from freezing even exists [32,33]. Contributing to the uncertainty was the fact that most studies correlating sugars with freezing tolerance measured sugars in whole plants or major parts of plants such as leaves, roots or crowns. But, it has been shown that whole plant death, at least from freezing, is a result of the death of specific regions of plants and even specific cells [34-41]. If sugars are concentrated in specific regions as suggested by Canny [42] this could reduce the freezing point

significantly in those locations. If higher concentrations of sugar were within a region(s) of the plant that is crucial for whole plant survival then while freezing point depression would certainly not be the only protective mechanism, it could be a significant means by which whole plants survive abiotic stress.

Sugars have also been cited as a means to help prevent cell plasmolysis. By determining kill temperatures of shrinking cells in solutions varying in solute concentration Williams [43] found a minimum volume to which cells were reduced before being killed (see Levitt [21] for a detailed review of frost plasmolysis). While it is clear from a multitude of studies that freezing damage to plant cells is the combined result of many injuries (e.g. oxidative damage, membrane phase changes, membrane fusion, break-down of transmembrane gradients, to name a few), plant cells can use sugars, synthesized during cold acclimation or from hydrolyzed fructan, to resist cell volume reduction by changing osmotic pressure. A study of the relationship of cell volume to freeze damage in wheat not only confirmed that minimum cell volume is a significant contributor to tissue death but showed that wheat changed its lethal cell volume size by altering membrane properties which in turn lowered its kill temperature [43]. Because of the relationship of sugars to drought tolerance [44] similar protective mechanisms for fructan with regard to dehydration stress were proposed. One such mechanism is that hexose sugars from hydrolyzed fructan could lower the water potential of intracellular liquid and allow continued leaf expansion in drought periods [3,45,46]. This is identical in principle, at least, to protection from damage during freezing caused by minimum cell volume described by Williams [43].

2.2. Role of fructan in subzero acclimation

Trunova [47] reported that fructan decreased while simple sugars (mostly fructose) increased when wheat plants were frozen under mild conditions (-3°C) Concomitant with this redistribution of carbohydrates was an increase in freezing tolerance beyond that achieved when plants were cold acclimated at temperatures above freezing. This newly discovered component of winter hardiness [Trunova, 47, cites Tumanov with this discovery in 1931] was called "second phase hardening" in contrast to first phase hardening that occurred at temperatures just above freezing. Recently second phase hardening has been referred to as "subzero acclimation" [37].

Olien found that fructan hydrolysis and a concomitant increase in hexose sugars during subzero acclimation was more pronounced in rye (the most winter hardy cereal crop) than it was in barley and furthermore that the increase in hexose sugars was primarily in the apoplast. Earlier he had proposed a mechanism of freeze injury called adhesion [48] which is a result of a hydrophilic compound such as a cell wall or membrane competing with

ice for liquid water at an interface [49]. In plants, as freezing progresses and dehydration causes cells to shrink, adhesions to walls and membranes can cause significant damage to cells that is histologically distinct from desiccation injury. Olien proposed that hexose sugars from hydrolyzed fructan are released into the liquid interface during freezing in hardy plants. This would increase the chemical potential of the liquid interface which would induce melting and either prevent or relieve adhesions.

Conflicting correlative studies [19,26,50] made it clear that the role of sugars and fructan in protection from abiotic stress was not a simple one [51]. While most correlative studies with grasses report positive correlations of fructan with freezing tolerance [52], Hendry [6] calls into question the relationship of fructan with freezing tolerance within 130 species of the less freezing tolerant Sheffield flora. Pollock et al [32] report no correlation between freezing and soluble carbohydrate in 2 *Lolium perenne* cultivars. Livingston et al. [53] reported that the tolerance of 23 oat genotypes to freezing under controlled conditions was correlated with the amount of smaller (DP<6) fructan and not with fructan DP > 6. However, in wheat, triticale and several rye cultivars, high DP fructan was more closely correlated to freezing tolerance than low DP fructan [19]. The relationship of fructan to abiotic stress tolerance is clearly more complicated than originally supposed and likely involves differences in size, and structure (described below) as well as localization [37] within tissue that is vital for survival of the whole plant [36].

3. Direct protective effects by fructan

3.1. Fructan localization within tissue that is vital for whole plant survival

While several mechanisms to explain the *indirect* role of fructan as a hexose reserve had been proposed, the localization of fructan exclusively in the vacuole [8,14,54] made a *direct* role for fructan in protection of the plasma membrane from freezing somewhat problematic. It did, however, leave open the possibility that fructan may protect the tonoplast from damage. Livingston and Henson [31] compared fructan and its hydrolysis products in crown tissues of oat and confirmed that a mild freeze increased apoplastic hexoses but also somewhat unexpectedly that fructan itself increased in the apoplast beyond levels that could be explained by simple membrane rupture. Fructan was also found in guttated liquid [31] confirming the presence of fructan in the apoplast of cold acclimated plants. Zuther et al. [16] demonstrated long distance transport of DP3 fructan via the apoplast (phloem) in potato, albeit this was in a transgenic system that does not occur naturally in potato. Wang and Nobel [55] demonstrated phloem transport of small fructans in leaf tissue of *Agave deserti.* The presence of fructan in the apoplast provides support for the hypothesis that fructan could involve a more direct role in

protection of tissues from freezing/dehydration injury either instead of or in addition to an indirect role as a hexose reserve.

Since membrane distortion and rupture is a major result of abiotic stress leading to cell death, artificial membranes have been the focus of recent studies on the relationship of various carbohydrates to membrane stability.

3.2. Importance of water in membrane integrity

Many investigations on the protective effects of sugars have used liposomes as comparatively simple model systems to elucidate the physical details of membrane protection during dehydration induced by freezing or drying. Water plays an essential role in the formation of bilayers from lipids and in membrane stability. Different parts of the lipid headgroups interact with water through H-bonding and ensure spacing of the lipid molecules in the liquid crystalline state [see 56 for a comprehensive review]. When membranes are dehydrated, water molecules that help to maintain this spacing between the lipid headgroups are (partially) removed, allowing a closer approach of the lipid molecules. This leads to an increase in van der Waals interactions between fatty acyl chains and to an increase in the gel to liquid-crystalline phase transition temperature (T_m) of membrane lipids by as much as 70°C [57]. This elevation of T_m in dry membranes is especially important if membrane lipids have a sub-ambient T_m under hydrated conditions, as is the case for biological membranes. When T_m increases above the ambient temperature, lipids will undergo two phase transitions, one from liquid-crystalline to gel phase during drying and one from gel to liquid-crystalline phase during rehydration [see 58,59 for reviews]. These phase transitions result in transient leakage of soluble cell contents through the membrane [60,61]. Therefore, in liposomes, damage during drying and rehydration is commonly determined as the leakage of a soluble marker such as carboxyfluorescein (CF) from the interior of the lipid vesicles. This leakage is thought to be due to inhomogeneities in the membrane during the phase transition [62], because of the coexistance of gel and fluid phase lipids that results in packing defects and increased permeability [63].

3.3. Sugar mediated protection of liposomes

In contrast, when membranes are dried in the presence of sufficiently high amounts of sugars such as trehalose or sucrose, leakage of soluble content from liposomes can be largely prevented [64-66]. The water replacement hypothesis suggests that sugar molecules prevent the close approach of lipids during dehydration through H-bonding interactions between sugar OH-groups and lipid headgroups. This prevents dehydration-induced increase in T_m [58] and consequently phase transitions and solute leakage. Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy

(NMR) have provided evidence for such interactions between disaccharides and phospholipid headgroups, mostly at the level of the P=O moiety of phosphatidylcholines [67-69].

In addition to phase transitions, leakage is also triggered by fusion of liposomes under conditions of severe water loss. In most cases, fusion of liposomes is accompanied by formation of transient pores in membranes, which allow soluble molecules to diffuse out of vesicles [70,71]. Most sugars form glasses (vitrify) during drying at ambient temperatures [see 72,73 for reviews]. Due to the high viscosity of such glasses, all processes that require diffusion are slowed down to a degree that makes them stop on the scale of human observation [74]. Vitrification during drying will therefore fix the position of liposomes in the glassy matrix, so that the close approach of vesicles necessary for fusion is prohibited. Obviously, the effectiveness of this mechanism depends on the physical stability of the glass. The melting temperature of a sugar glass (glass transition temperature; T_g) is a convenient and often used measure of glass stability [73]. Raising the temperature of a vitrified sample above the T_g of the system leads to increased fusion and leakage from liposomes [66].

3.4. Membrane protection by polysaccharides

On the other hand, using sugars with increasing T_g results in reduced liposome fusion in the dry state, especially at elevated temperatures [75]. In general, T_g increases with molecular weight of the solute [76]. This is also true for oligosaccharides such as raffinose family oligosaccharides (RFO) [77], fructo-oligosaccharides [78,79], and malto-oligosaccharides [80]. Water is an effective plasticizer of sugar glasses, although the degree of plasticization varies between different di-, oligo-, or polysaccharides [78,80,81]. In general, a sugar with a higher T_g will vitrify at a higher water content at a given temperature during drying. Therefore, a higher T_g will be beneficial during drying, because it allows vitrification and prevents fusion at an earlier stage of the drying process.

Polysaccharides could be expected to be good protectents for liposomes during drying, because in the dry state, all polysaccharides that have been investigated in this regard showed high T_g values (fructans 154°C [79]; hydroxyethyl starch (HES) > 100°C [61,83]; dextran >100°C [82]). Therefore, they are expected to be efficient protectants against liposome fusion in the dry state. Such protection has actually been shown during air-drying for HES [61,83], dextran, and fructan [84].

However, HES and dextran did not, or only to a very small degree, protect liposomes against leakage [61,83-85]. The reason for this could be found in the inability of HES [61,67,83,85], and dextran [86,87] to depress T_m in dry

membranes. This has been related to the inability of these polysaccharides to H-bond to the lipid headgroups [61,65,67,83,85].

3.5. Membrane protection by fructan

The class of polysaccharides that has been most thoroughly investigated with regard to their protective effects on liposomes during drying are fructans. Both a plant inulin (from chicory roots) and a bacterial levan (from *Bacillus subtilis*) have been shown to protect liposomes from leakage during freeze-drying or air-drying [84,85]. Commercially available Chicory inulin is a mixture of polysaccharides with a degree of polymerization (DP) between 10 and 30, corresponding to molecular masses between approximately 1600 and 5000 [85]. During freeze-drying, the presence of chicory inulin in phosphatidylcholine liposome preparations reduces the degree of leakage after rehydration [85]. This protective effect is related to a depression of T_m in dry membranes compared to liposomes dried without fructan. By FTIR it was shown that inulin establishes H-bonds to lipid P=O, despite its large size [85]. This indicates that steric factors can be overcome even by large molecules to enable the insertion of at least part of the polysaccharide into the lipid headgroup region. It was shown in the same study that HES was not able to interact with P=O groups in the dry membrane or depress T_m, under identical experimental conditions. This indicates that fructan has specific structural properties that this glucan does not possess. During slow air-drying, chicory inulin provides no protection to liposomes [88]. This is due to the low solubility of chicory inulin which, therefore, precipitates during the slow drying process, while during freeze-drying inulin is immobilized during the freezing step. Inulins with a lower DP ($\leq$ 10), which are more soluble, do not precipitate during air-drying and provide protection to liposomes [84,88].

Levan isolated from *Bacillus subtilis* has a DP of about 125, corresponding to a molecular mass of approximately 25000 [89]. Although this fructan has a much higher DP than chicory inulin, it also has a much higher solubility. This is reflected in the fact that it will not precipitate from solution during air-drying and that it protects liposomes from leakage and fusion during drying and rehydration [84]. It has been shown by x-ray diffraction measurements that levan is located between liposomes in the dry state, thus enabling both encasement in a glassy matrix and direct interactions with membrane lipids [84]. In contrast to HES and dextran, the presence of levan resulted in a clear depression of T_m and increased mobility of fatty acyl chains in dry membranes, as determined by FTIR and NMR spectroscopy [87]. NMR measurements also indicated a strong immobilization of the headgroup both at the P=O and the choline level in the presence of levan [87] and recent FTIR analyses provided evidence for limited H-bonding of levan to lipid P=O groups [90].

3.6. The effect of fructan size and structure on membrane protection

To gain further insight into the physical mechanisms and structural determinants of membrane protection by fructans, we compared the effects of different structural families of oligosaccharides on liposomes during drying. It had been suggested earlier that oligosaccharides show reduced protection for liposomes during drying, compared to sucrose, and that strongly reduced protective effects can be expected above DP 3 [83,91]. Our analyses showed that this is true for manno- and malto-oligosaccharides, while fructans and RFO show the opposite behavior, i.e. increased protection with increasing DP [75,88,92]. Also, a cyclic inulin of DP 6 (cycloinulohexaose) showed good protection of liposomes during freeze-drying [93], indicating that it may be specific structural features of oligosaccharides that determine their efficacy as membrane stabilizers during drying.

In general, one would expect better protection against fusion from longer oligosaccharides, because of the increase in T_g. This expectation is borne out by RFO [75,77], and malto-oligosaccharides [88], which show increased protection against fusion with increasing DP. However, protection against fusion decreases with increasing DP in the case of inulins [88].

The effects of sugars on T_m follow a different pattern than the effects on fusion. Inulins (up to DP 5) show no effect of DP on T_m [92], RFO (up to DP 5) lead to a slight increase in T_m with DP [75], while malto-oligosaccharides (up to DP 7) and manno-oligosaccharides (up to DP 6) lead to progressively stronger increases in T_m with DP [88,92]. The shift in spectral position of P=O vibration in FTIR spectra, indicating H-bonding between sugar and lipid headgroups shows no effect of DP for inulins, a slight reduction for RFO, but strong reduction for malto- and manno-oligosaccharides, in general agreement with the T_m data [75,88,92].

These data suggest that specific structural features of different oligosaccharide families determine their dramatically different abilities to H-bond to lipid headgroups in membranes in the dry state. There are various levels at which the structure of oligosaccharides could differ and thereby influence interactions with membrane surfaces. These could include different degrees of structural flexibility around the glycosidic bonds, chair-boat conformational transitions, and differences in exposed hydrophobic surface area that might facilitate interactions with membrane lipids. There is some indication in the literature that structural flexibility may be the crucial factor that distinguishes the different oligosaccharide families. Evidence for this hypothesis comes for instance from molecular dynamics simulations that show large differences in oligosaccharide structure between gas phase and solution, indicating a major influence of H-bonding interactions on oligosaccharide structure [94,95,96].

The main structural difference between inulins and other oligosaccharides is that inulins are mainly oligofructoses (except for one terminal glucose unit) composed of rather flexible furanose rings [97,98], while other oligosaccharides, e.g. galactose, glucose or mannose, are composed of more rigid pyranose rings [98]. Therefore, the higher flexibility of the furanose ring may counterbalance the negative steric effects of increasing DP in inulins, leading to an independence of inulin-membrane interactions from size. In more rigid oligosaccharides, on the other hand, negative steric effects dominate the size dependence of these interactions.

This leaves open the question of why different pyranose-based oligosaccharides behave differently with available evidence suggesting a significant contribution of linkage type to the structural flexibility of such sugars. Of the pyranose-based oligosaccharides, RFO show the highest degree of interaction with dry lipids [75]. RFO are 1,6 linked carbohydrates and it has been shown recently that this linkage type affords oligosaccharides additional flexibility compared to 1,4 linked oligosaccharides [99]. This is related to the fact that 1,6 linkages involve three dihedral angles, while 1,4 and 1,3 linkages only involve two dihedral angles, contributing different amounts of structural flexibility [100,101]. Sugars with a 1,6 linkage show a strong influence on structure of H-bonding to water molecules [95], implying the ability to adapt their conformation to optimize H-bonding to lipid molecules in the absence of water. Similarly, differences in the effects of malto- and manno-oligosaccharides may be related to the higher flexibility of the α-glycosidic linkage compared to the β-glycosidic linkage [101,102]. While the type of glycosidic linkage will certainly have an effect on the mechanical properties of sugars, there are no simple rules to link the two properties. For instance, different β 1→4 linked polysaccharides vary in their mechanical properties and bond flexibility between highly rigid (e.g. chitin, cellulose) and highly flexible (e.g. xylan, hyaluronan) [96], indicating that, depending on other structural features, the same glycosidic bond can result in contrasting mechanical properties for different sugars.

An additional degree of flexibility is inherent in most sugars from the ability for transitions between boat and chair conformations. These conformational transitions have been investigated using atomic force microscopy [see Brant, 103 for a review] which has shown that different oligosaccharides require different amounts of energy for such transitions [104-107]. It is unclear, whether such transitions occur during drying, but forces that act on solute molecules during drying may be large enough to force such conformational transitions, at least in some oligo- and polysaccharides. Clearly, more research is needed to understand the details of structural aspects of sugar-lipid interactions within and between membranes.

4. Transgenics

4.1. Examples of information obtained from transformed systems

An ideal means to investigate the physiological relationships of soluble sugars to abiotic stress tolerance, is the possibility of establishing non-host metabolic pathways by gene transfer techniques in nearly isogenic backgrounds. An example of this kind of investigation is the expression of *Escherichia coli* mannitol-1-phosphate dehydrogenase in tobacco which led to the accumulation of mannitol [108] and improved salt tolerance [109]. Another example is an increased drought tolerance of transgenic tobacco plants [110] into which trehalose-6-phosphate synthase was transferred from baker's yeast.

In some cases, interesting characteristics of metabolites or enzymes were discovered from transgenic experiments. For example, it turned out that compounds like the sugar alcohols mannitol or D-ononitol, which were believed to be metabolically inert in tobacco, may reduce sink capacity by interfering with respiration or glycolysis [111]. For the enzyme inulinsucrase from *Aspergillus sydowi* it could be demonstrated, using various hosts for transgene expression, that product specificity was strongly influenced by the environment [112].

4.2. Direct physiological effects in transformed plants, or pleiotropy?

The first cloned genes encoding fructan biosynthetic enzymes were bacterial levansucrase genes from *Bacillus subtilis* [113], *Zymomonas mobilis* [114] and *Erwinia amylovora* [115] and an inulinsucrase from *Streptococcus mutans* [116]. All four genes and homologs from other bacterial species [reviewed by 117] have been expressed in transgenic plants. Expression of the *Bacillus* sacB gene [51] and the *Zymomonas* levU gene [118] reportedly improved abiotic stress tolerance. However pleiotropic effects like growth retardation, necrotic lesions or sterility have been reported in the case of bacterial levansucrase expression as well as in transgenic production of non-host sugar alcohols or trehalose. In addition to pleiotropic effects, concentrations of the foreign metabolites were often very low. These aspects have all raised questions as to whether observed effects on stress tolerance could be indicators of genuine physiological functions of the metabolites under consideration [117,119].

Apart from the studies mentioned, the question of a role of fructans in stress tolerance remained largely unanswered – partly because plants were only tested under controlled and sometimes artificial conditions such as polyethylene glycol mediated osmotic stress. However, Konstantinova et al. [120] field-tested transgenic tobacco plants accumulating high levels of proline

or producing levans and glycine betaine. The oriental-type cultivar Nevrokop 1146 of *Nicotiana tabacum* did not show phenotypic alterations following transformation with the *Bacillus subtilis* sacB gene and was more freezing tolerant under controlled as well as under field conditions. Unfortunately, fructan concentration in the transgenic lines was not determined, but an increase in freezing tolerance after a cold treatment, probably due to accumulation of the substrate sucrose, was reported [120].

In a follow-up study, transgenic plants were analyzed for oxidative damage at chilling and sub-zero temperatures [121]. It turned out that malondialdehyde levels, which are indicative of lipid peroxidation, are lower in fructan producing tobacco lines. Although interactions of fructans with lipids are well established (see above), protection by fructan against peroxidation is not conclusive, and thus the observed effect may argue again for pleiotropic responses to sacB expression. The sacB transgenics had elevated levels of hydrogen peroxide at normal temperatures, and this might in fact be the reason for improved tolerance, because hydrogen peroxide has been reported to stimulate abiotic as well as biotic stress responses in plants [122,123]. Unfortunately, statistical significance of the rise in hydrogen peroxide in sacB transformants was not tested.

4.3. Transformation with fructosyl transferases

Plant genes encoding fructosyltransferases became available in the mid-1990s [124-126] and were subsequently used for plant transformation. Among other discoveries these studies demonstrated that sucrose dependent fructosyltransferase 1-SST produces not only the trisaccharide 1-kestose but also higher homologs *in planta* [127-128]. In addition, it was confirmed that inulin synthesis in dicotyledonous plants needs only two enzymes, 1-SST for initial fructan synthesis from sucrose and 1-FFT [128] for elongation of the polymer, as hypothesized by Edelman and Jefford [8]. But important questions still remain. For example, it is not clear, why fructan levels in transgenic plants are generally much lower than in native fructan producers. It has been suggested that endogenous invertase activities of the non-fructan host systems interfere with fructan production [117]. However, this probably does not apply to potato tubers, where invertase activity is low during loading [129], while fructan accumulation is still only about 5% of the amount of starch in tubers [128].

That not all questions could be answered has caused some disproportional criticism. The fact that expression of 1-SST in transgenic plants led to accumulation of not only 1-kestose but also oligofructans up to DP7 does not argue against successful ectopic expression of 1-SST, which 'by definition' would only produce trisaccharides [117], but demonstrates that acceptor- and

donor-specificities of fructosyltransferases are not as strict as initially hypothesized.

Many investigations of fructosyltransferase specificity made use of the methylotrophic yeast *Pichia pastoris*, which allows convenient production and purification of foreign enzymes [130-137]. These studies revealed that the specificity of 1-SST and 6-SFT are determined by the N-terminal large subunit of the protein [132] and that transferase and hydrolytic activities can be separated [133]. However, the *Pichia* system is not always reliable. For example, the barley 6-SFT gene expressed in *Pichia* yielded an enzyme that had additional 1-SST activity, which is not the case when the enzyme is purified from barley [130]. Whether such deviations from normal activities results from expression of the enzymes as secreted proteins in *Pichia* is not clear. Nevertheless, the system allows rapid identification of fructosyltransferase activities, which is not possible for bacterial expression systems, and is therefore frequently used for cloning fructosyltransferase genes.

4.4. Low temperature response of fructofuranosidases

Genes encoding 1-SST and 6-SFT from wheat have been identified based on sequence homology to β-fructofuranosidases and expression in *Pichia* [138]. Both genes are responsive to low temperatures with 1-SST being transcriptionally induced in leaf and crown tissue during cold acclimation. Expression of 6-SFT in leaves during subzero acclimation discriminates frost tolerant and snow mold-resistant cultivars. While 6-SFT is down-regulated in frost tolerant genotypes, it remains high in snow mold-resistant lines, when temperatures fall below zero [138]. Expression of the genes correlates with fructan levels, which decline when temperatures fall below zero. A reduction in the amount of fructan in crown tissue under prolonged stress conditions has also been reported for the New Zealand grass *Festuca novae-zelandiae* during water deficit [139]. It may, therefore, appear questionable whether fructans provide direct protection under conditions of severe stress. As mentioned above, fructans could protect tissue indirectly by providing carbon and energy for the synthesis of other cryo- and osmo-protectants.

There is clear evidence for a metabolic regulation of fructan synthesis in the cold. It has long been known that fructan synthesis is induced by high sucrose concentrations in leaves of grasses [14] and *Asteraceae* [125]. Fructans therefore, could accumulate in the cold or at mild water deficit simply because of high sucrose levels, probably as a result of lowered respiratory activities in sink tissues. Fructosyltransferase genes, especially the 1-SST gene as pacemaker for fructan synthesis, are induced by high sucrose [140-141]. Expression of 1-SST in various transgenic plant systems has also revealed that fructan synthesis may depend entirely on high sucrose concentrations. While expression in sugar beet led to high levels of fructo-oligosaccharides [142],

levels were considerably lower in all other systems that had lower sucrose concentrations (see above). So it cannot be ruled out that fructan accumulation in the cold simply serves as sink for assimilates that are not consumed because of low metabolic activity.

However, another set of transgenic plants argues against this possibility. Perennial ryegrass *(Lolium perenne*) was transformed with the 1-SST and/or 6-SFT genes from wheat mentioned above. Expression of either of the two caused a 3- to 15-fold increase in fructan content and a higher freezing tolerance at –8 and –12°C [143]. Freezing tolerance was tested under laboratory conditions using a well established test that takes electrolyte leakage from leaves as a measure for cell damage. Under these conditions, fructans are not degraded prior to low temperature exposure, thus ruling out the possibility that fructan breakdown is needed for elevated frost tolerance. In the transgenic plants, sucrose levels were identical to the wild-type, so protective effects of sucrose cannot explain the higher tolerance of the transgenics.

5. Future

Experiments with model systems and with transgenic plants have contributed to the discovery of cause and effect relationships between fructan and resistance to abiotic stress. However, rather than establishing an unequivocal relationship, much of the research has revealed a complex interaction of colligative, and non colligative mechanisms, mediated by genetic factors, all of which are likely to be important in various tissues [39,144] at different times [37] during acclimation, freezing and drought. Clearly, more information on transformation systems is needed to answer questions with regard to fructan structure such as: is there a change in the average chain length of the fructans, when 1-SST is over-expressed? What fructans accumulate in 6-SFT expressing ryegrass? It has been reported that a typical 6-SFT activity is not present in *Lolium perenne*, which produces mainly inulin- and neo-kestose-type fructans [145]. The expression of 6-SFT would thus establish a new pathway of fructan synthesis. The transgenic ryegrass system offers a chance of studying the influence of chain length as well as structure of fructans on possible protective effects. Purification of fructan isomers of varying size and structure for studies with model membranes during drying and freezing [146] also offers the possibility of providing a better understanding of the structural determinants for membrane stabilization. This knowledge may enable the use of particularly effective fructans e.g in the biostabilization of pharmaceutical products and together with transgenic approaches or marker-assisted selection may lead to crop plants with improved performance of the most yield-limiting component in crop production, abiotic stress.

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