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In Concert: Orchestrated Changes in Carbohydrate Homeostasis Are Critical for Plant Abiotic Stress Tolerance

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The sessile lifestyle of higher plants is accompanied by their remarkable ability to tolerate unfavorable environmental conditions. This is because, during evolution, plants developed a sophisticated repertoire of molecular and metabolic reactions to cope with changing biotic and abiotic challenges. In particular, the abiotic factors light intensity and ambient temperature are characterized by altering their amplitude within comparably short periods of time and are causative for onset of dynamic plant responses. These rapid responses in plants are also classified as 'acclimation reactions' which differ, due to their reversibility and duration, from non-reversible 'adaptation reactions'. In this review, we demonstrate the remarkable importance of stress-induced changes in carbohydrate homeostasis of plants exposed to high light or low temperatures. These changes represent a co-ordinated process comprising modifications of (i) the concentrations of selected sugars; (ii) starch turnover; (iii) intracellular sugar compartmentation; and (iv) corresponding gene expression patterns. The critical importance of these individual processes has been underlined in the recent past by the analyses of a large number of mutant plants. The outcome of these analyses raised our understanding of acclimation processes in plants per se but might even become instrumental to develop new concepts for directed breeding approaches with the aim to increase abiotic stress tolerance of crop species, which in most cases have high stress sensitivity. The latter direction of plant research is of special importance since abiotic stress stimuli strongly impact on crop productivity and are expected to become even more pronounced because of human activities which alter environmental conditions rapidly.

Keywords: Cold stress • Sugar transport • Vacuole • Fructans.

Abbreviations: AGPase, ADP glucose pyrophosphorylase; BvIMP, *Beta vulgaris* INTEGRAL MEMBRANE PROTEIN; BvTST2.1, *Beta vulgaris* TONOPLAST SUGAR TRANSPORTER 2.1; DP, degree of polymerization; ERDL6, EARLY RESPONSIVE TO DEHYDRATION 6-LIKE 6; ESL1, EARLY-RESPONSIVE TO DEHYDRATION SIX-LIKE 1; 1-FEH, FRUCTAN 1-EXOHY DROLASE; 1-FFT, FRUCTAN: FRUCTAN FRUCTOSYLTRANSF ERASE; FNR, ferredoxin-NADP$^+$ oxidoreductase; MFS, major facilitator superfamily; MST, monosaccharide transporter; NTRC, NADPH-dependent thioredoxin reductase C; OPPP, oxidative pentose phosphate pathway; PCR4, PCR CLONE 4; PET, photosynthetic electron transport; pmf, proton-motive force; ROS, reactive oxygen species; 1-SST, SUCROSE: SUCROSE FRUCTOS YLTRANSFERASE; SUT4, SUCROSE TRANSPORTER 4; SWEET, SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER; TRX, thioredoxin; TST, TONOPLAST SUGAR TRANSPORTER; VGT1, VACUOLAR GLUCOSE TRANSPORTER 1.

Sugars are Uniquely Suited to Protect Cellular Function from Stress-Induced Reactive Oxygen Species (ROS) Production

In this review we will focus on the importance of changes in sugar homeostasis allowing plants to resist situations of both high light and low temperatures. Thus, given that sugars are involved in the cellular response to both types of stress stimuli, we have to reason that plants exposed to these challenges exhibit similar pathophysiological stress responses with the potential to damage important endogenous structures and, by this, to impair pivotal cellular processes.

Both high light and low temperature are environmental factors that change their amplitude rapidly. Plant responses to low temperature and high light are tightly interacting since both metabolic and molecular acclimations to low temperature are strictly dependent on light, which is the driving force for accumulation of carbohydrates involved in cold tolerance (Catalá et al. 2011, Lee and Thomashow 2012, Hörmiller et al. 2017). However, both conditions provoke a common metabolic response in plants, namely an increased generation of ROS (Suzuki and Mittler 2006, Alboresi et al. 2011, Choudhury et al. 2017). Thus, ROS accumulation upon onset of stress has to be set in the scenario of plant acclimation reactions.

At first glance, it might be surprising to see that onset of high light or low temperature leads to ROS accumulation in plant cells beyond the level which is favorable for cellular functions (Foyer and Noctor 2013, Baxter et al. 2014). However, taking the thylakoid-located photosynthetic electron transport (PET) chain as the main source for ROS synthesis in leaves, it becomes clear why both low temperature and high light induce ROS synthesis. Stromal-located enzymatic reactions, which consume

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most reducing equivalents and energy in the form of ATP, either slow down significantly in the cold, while PET rates stay relatively high, or cannot keep pace with excess electrons liberated as a result of excess light exposure. Accordingly, under those challenging conditions, photosynthesis changes from a beneficial process into a potentially dangerous one because of its latent capacity to produce harmful ROS (Foyer et al. 2009).

In fact, ROS are not exclusively synthesized at the thylakoid-located PET chain, but also at the mitochondrial electron transport chain, either by cell wall-located peroxidases, plasma membrane-located NADPH oxidases, the peroxisomal-located glycolate oxidase during photorespiration or in glyoxysomes during fatty acid degradation (Dietz et al. 2016). Thus, this large number of processes involved in ROS production fully explains why nearly all challenging environmental factors, affecting one or other of these complex pathways, might be causative for ROS accumulation in plant cells (Foyer and Noctor 2013).

What Makes Sugars So Relevant for Stress Tolerance?

Although we know today that all types of pro- and eukaryotic cells require a certain level of ROS for internal signaling processes and that ROS in cells are even essential against certain pathogens (Schieber and Chandel 2014), stress-induced ROS accumulation exceeding critical concentrations is detrimental for cell function. This is because ROS, especially in the form of singlet oxygen and various free radicals, effectively attack DNA and RNA molecules, modify soluble and membrane-bound proteins, modify prosthetic groups required for proper protein function and can cause non-enzymatic lipid peroxidation, which in particular impairs membrane function and integrity (Sharma et al. 2012). In fact, several independent functions make sugars so efficient at withstanding stress situations:

1. Sugars as highly hydroxylated soluble molecules have per se superior function as stabilizers of membranes and proteins in cells exposed to different stress stimuli (Hoekstra et al. 2001, Hengherr et al. 2008). However, with respect to stress-induced ROS production, during low temperature and high light situations, it is of particular relevance that sugar molecules themselves are prone to be attacked by ROS at their many HO–C–H linkages (Morelli et al. 2003). Moreover, when sugar molecules are located in the immediate vicinity of membranes, they may directly scavenge hydroxyl radicals originating from damaged lipids or lipid peroxidation. Accordingly, a first conclusion might be that the higher their concentrations in plant cells is the more efficiently sugars can serve as ROS quenchers. However, we will illustrate below that such a simplistic view is not completely comprehensive. For instance, for achieving frost tolerance in higher plants, it seems rather important that a specific type of sugar is located in specific cellular compartments (see below).
2. Besides their function as ROS scavengers, in the case of frost temperatures, high sugar concentrations might contribute to the stabilzation of the osmotic cell potential required to decrease the freezing temperature. Additionally, it has been known for decades that sugars stabilize membranous structures at low temperatures (Lineberger and Steponkus 1980). Particularly at temperatures below 0°C, sugars enable the induction of a process termed 'vitrification' which occurs at plasma membrane and intracellular membrane surfaces (Wolfe and Bryant 1999, Hincha et al. 2006). During this process, frost-induced limited availability of water molecules is compensated by highly hydroxylated sugar moieties, which lead to maintenance of membrane integrity.
3. In addition, glucose and fructose feed, after being phosphorylated, directly into the oxidative pentose phosphate pathway (OPPP). The latter pathway is, due to the regeneration of NADPH, of particular importance for tolerance against ROS in both animal and plants cells (Couemal and p2006, Patra and Hay 2014). Since in plants the OPPP is present in both plastids and the cytosol (Sweetlove and Fernie 2013), redox equivalents generated within this pathway are thus available for ROS detoxification at various cellular locations, e.g. via NADPH-dependent ascorbate peroxidase reactions (Caverzan et al. 2012) or via glutathione reductase activity representing a critical element of the Halliwell–Asada pathway (Mhamdi et al. 2010).
4. Sugars represent the only source of substrates that lead to the biosynthesis of sugar alcohols. The latter group of molecules, comprising, for example, sorbitol or mannitol, are also known as very efficient ROS scavengers, mainly because they exhibit a high water solubility and possess even more hydroxyl groups than their sugar precursors (Smirnoff and Cumbes 1989). This impressive ROS-scavenging activity of sugar alcohols is so strongly pronounced that some plant pathogenic fungi even produce these compounds to avoid the defense attack by the infected plant (Upadhyay et al. 2015).

How Plant Cells Ensure Carbohydrate Synthesis Even Under Stress Conditions

According to the highlighted examples above on the protective function of sugars, it is not surprising that defects in starch or sucrose metabolism impair the ability of plants to resist both high light- and low temperature-induced stress situations (Wanner and Junttila 1999, Häusler et al. 2014, Schmitz et al. 2014). In this context, it is remarkable to observe that plants, challenged by these independent stress stimuli which are expected to cause impaired carbon assimilation efficiency, are in fact able to maintain high sugar concentrations (Wormit et al. 2006, Poschet et al. 2011, Jänkänpää et al. 2012, Klemens et al. 2013, Klemens et al. 2014, Le Hir et al. 2015). Starch turnover in leaves is essential for the provision of sugars required for various cellular processes in the next night phase (Streb and Zeeman 2012). Accordingly, it is mandatory for plants to maintain starch turnover and thus to ensure that sugar levels stay high or can become even further increased after onset of challenging environmental conditions. Recently described regulatory processes connected to starch synthesis as well as to the ability of plants to tune down sugar sensing under selected conditions might contribute to this process (see below).

The rate-limiting step of transitory starch accumulation is the reaction catalyzed by the enzyme ADP glucose pyrophosphorylase (AGPase). Allosteric activation of AGPase by an increasing

3-phosphoglycerate/Pi ratio, which is of particular importance for a consistent adjustment of carbon flux into starch during permanently altering light intensities, was the first mode of activity regulation identified for this enzyme (Preiss 1993). However, an increasing body of literature now reveals that substantial post-translational protein modifications both on the AGPase and on starch-mobilizing enzymes are important in controlling the level of transitory starch (Kötting et al. 2010). In this context, both redox regulation by thiol–disulfide modulation and reversible protein phosphorylation have now been linked to regulation of starch turnover (Geigenberger 2011, Streb and Zeeman 2012).

However, to understand how starch turnover is maintained under excess light or low temperature conditions, we first and foremost have to consider typical changes in amounts of cellular components putatively contributing to stabilization of reasonably high starch turnover rates: after onset of high light, a typical over-reduction of the complete PET chain not only causes chloroplast-located ROS formation but also provokes a significant increase in stromal NADPH levels (Endo et al. 2005). Interestingly, AGPase is grouped among the light-activated enzymes and both thioredoxin (Trx) *f*1 and the stromal-located NADPH-dependent thioredoxin reductase C (NTRC; characterized as harboring an NTR and a Trx domain) are capable of reducing AGPase by thiol modification (Michalska et al. 2009). Thus, both electrons accumulating in the thylakoid electron transport chain and reducing equivalents derived from accumulating stromal NADPH can be used under high light stress to activate AGPase. Interestingly, NADPH as the redox donor for the NTRC reaction (Geigenberger 2011) is not only produced by ferredoxin-$NADP^+$ oxidoreductase (FNR) in (high) light but is also a product of the stromal-located OPPP. Accordingly, since accumulating cellular sugars can potentially feed into the plastidic OPPP, a further feed-forward signal for AGPase activity can be generated by these solutes under cold conditions, independent of the PET chain.

However, in any case, starch biosynthesis depends upon the maintenance of photosynthetic CO_2 fixation, the basis for de novo carbohydrate synthesis. Interestingly, under both stress conditions, i.e. excess light and cold temperature, an accumulation of soluble sugars in leaves is typical (Wulff-Zottele et al. 2010, Hedrich et al. 2015) and provokes a specific problem plants have to deal with. That is, high cytosolic sugar contents usually signal to tune down photosynthesis which generally decreases the expression of a plethora of genes with relevance for CO_2 fixation (Eveland and Jackson 2012). However, in the case of low temperature stress when the photosynthetic rate has to be kept high, it was shown that the sugar signaling system does not work in that generally accepted manner. This is demonstrated by the observation that during mid-term transition from acclimation to adaptation which takes place after a couple of days at low temperatures, newly developed leaves do not show the normally observed sugar-induced repression of genes involved in CO_2 fixation (Strand et al. 1997). Such insensitivity of the sugar-sensing system against rising levels of cellular sugar contents, which is far from being clarified on a functional level, allows plants to keep photosynthesis comparably high at low temperatures.

Examples of Intracellular Sugar Transport

Plants use sugars for an impressive large array of cellular functions. These functions comprise the provision of substrates for (i) oxidative phosphorylation; (ii) cellulose and hemicellulose biosynthesis; (iii) protein and lipid modifications; and/or (iv) cellular production of further primary or secondary metabolites. Due to this high diversity of pathways depending on sugars, it is not surprising to see that higher plants contain a wide number of different types of sugars at very individual concentrations. Generally, all sugars are highly soluble and have many hydroxyl groups. Nevertheless, the potential to serve as molecules efficiently assisting in acclimation to abiotic stress seems to depend on factors such as the molecular structure of a particular sugar, its individual concentration and the subcellular location at which it is accumulating. The main cellular reservoir for sugars is the vacuole (Martinoia et al. 2007, Martinoia et al. 2012) which, due to its large size, is the most suitable cell organelle for dynamic sugar storage. Accordingly, sugar import and export proteins located in the vacuolar membrane, the tonoplast, garner special attention for the discussion of stress-induced alterations in sugar homeostasis (**Fig. 1**).

In most plant species, three types of sugars contribute overproportionally to the overall sugar level, namely the disaccharide sucrose and the two monosaccharides glucose and fructose. In leaves, these three sugar species usually accumulate during the day, are consumed in the subsequent night phase and generally increase substantially after onset of abiotic stress stimuli such as high light or low temperature (Bläsing et al. 2005, Obata and Fernie 2012).

Within the cell, most of the sugars are located in the vacuole, and accumulation of sugars against a concentration gradient in this organelle depends upon the existence of a proton-motive force (pmf) in the form of a pH gradient (Rienmuller et al. 2012, Hedrich et al. 2015). Thus, since a pH gradient across the tonoplast is mandatory for proper cell function (Martinière et al. 2013), vacuolar import and export of sugars have to be mediated by different types of transport proteins. Having this energetic premise of mesophyll vacuoles in mind, it is not surprising to see that a large number of different tonoplast sugar transporters have been identified by various proteomic studies (Carter et al. 2004, Schmidt et al. 2007, Whiteman et al. 2008a, Whiteman et al. 2008b, Schulze et al. 2012).

However, so far, only two types of vacuolar sugar importers, namely the TONOPLAST SUGAR TRANSPORTERS (TSTs) (Wormit et al. 2006, Wingenter et al. 2010, Jung et al. 2015) and the VACUOLAR GLUCOSE TRANSPORTER 1 (VGT1) (Aluri and Büttner 2007) are characterized as exploiting the existing pmf for accumulation of glucose, fructose and sucrose. Phylogenetically, both types of transporters represent members of the monosaccharide transporter (MST) family from the major facilitator superfamily (MFS) group (**Fig. 2**).

The expression analysis of the *VGT1* gene, the only isoform of three paralogs in Arabidopsis so far analyzed experimentally, indicates high mRNA abundance in anthers but a low expression in leaves (Aluri and Büttner 2007). This organ-specific expression pattern correlates with the observation that *vgt1*

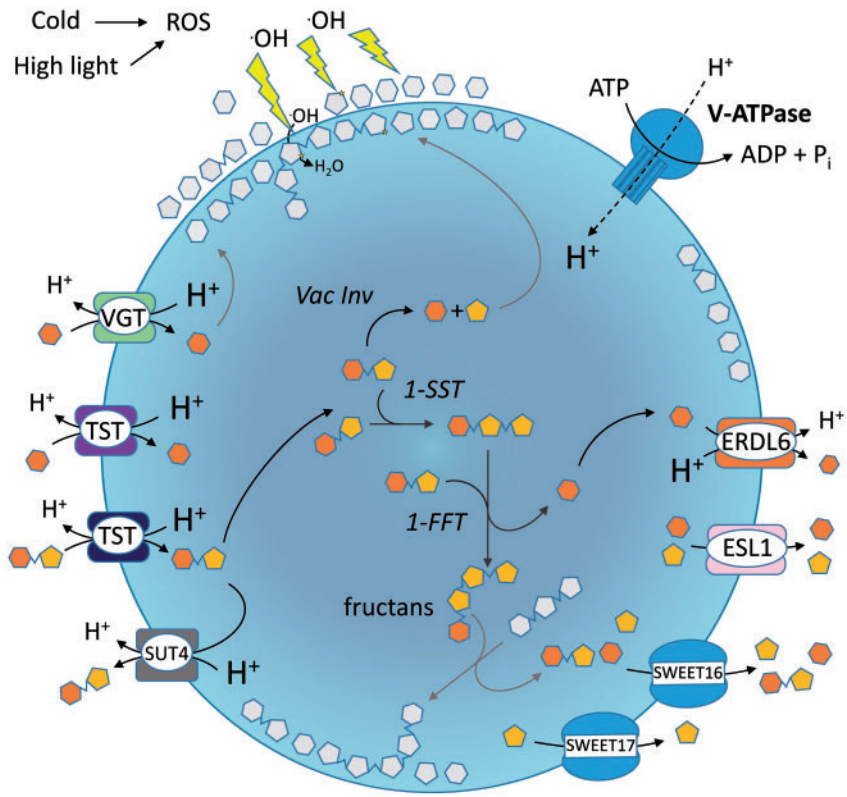

Fig. 1 Sugar transport proteins located in the vacuolar membrane adjust concentrations and composition of vacuolar sugars. The monosaccharides glucose (orange hexagons) and fructose (yellow pentagons) are imported in counterexchange with protons (H^+) via VGT (vacuolar glucose transporter) or TST (tonoplast sugar transporter) proteins representing subclasses of MST (monosaccharide transporter) family proteins. TST isoforms can also mediate the import of sucrose. The necessary H^+ gradient over the vacuolar membrane is established by an H^+-ATPase (V-ATPase). Release of sugars can be mediated by members of the Early-Responsive to Dehydration 6-Like proteins ERDL6 and ESL1, the sugar channel proteins SWEET16 or SWEET17, or sucrose/H^+ symporter SUT4-type proteins. Sucrose molecules can be hydrolyzed into glucose and fructose by vacuolar invertase (Vac Inv) or serve as building blocks for fructans synthesized by SUCROSE:SUCROSE FRUCTOSYLTRANSFERASE (1-SST) and FRUCTAN:FRUCTAN FRUCTOSYLTRANSFERASE (1-FFT). Like vacuolar monosaccharides and sucrose, fructans can serve as protectants of vacuolar membranes preventing bursting of the vacuole caused by stress-induced ROS-mediated peroxidation of membrane lipids.

loss-of-function mutants do not exhibit altered leaf sugar homeostasis but a slightly impaired flower development in comparison with wild-type plants (Aluri and Büttner 2007). In contrast to this, the absence of functional TST1 and TST2 proteins is in agreement with a nearly complete inability of cold-treated Arabidopsis plants to store both glucose and fructose in leaves (Wormit et al. 2006), clearly demonstrating that TSTs are the major vacuolar monosaccharide importer under these conditions. The latter conclusion receives further support from the observation that *TST* gene expression is cold and sugar induced (Wormit et al. 2006). Moreover, it has been shown that TST proteins from various species are prone to phosphorylation and that this post-translational type of protein modification is required for stimulation of its transport activity (Endler et al. 2009, Wingenter et al. 2011). The observation that selected peptides of TST1 and TST2 from Arabidopsis exhibit a significantly increased phosphorylation after cold treatment (Schulze et al. 2012) confirms the hypothesis that TST proteins are the major monosaccharide loaders in Arabidopsis mesophyll cells at low temperatures. Thus, in summary, it is not surprising that *tst1-2* loss-of-function plants exhibit a markedly reduced freezing tolerance as compared with wild-type plants (Klemens et al. 2014).

Besides glucose and fructose, most higher plants store the disaccharide sucrose in vacuoles (Neuhaus 2007). Although overexpressed TST1 from Arabidopsis exhibits sucrose transport capacity when analyzed electrophysiologically by whole-vacuole recording (Schulz et al. 2011), a nearly unchanged sucrose level in cold-treated *tst1-2* loss-of-function plants argues against an in vivo transport of sucrose by Arabidopsis TST proteins. However, in plant species that use sucrose either as a direct vacuolar storage compound or as a vacuolar precursor for synthesis of storage compounds, e.g. fructans, TST proteins may have been neofunctionalized to sucrose carriers during evolution or selective breeding history. In fact, high sucrose-storing plant organs such as the tap roots of sugar beet (*Beta vulgaris*) possess a unique TST ortholog named *Bv*TST2.1 which was shown to be extremely specific for sucrose transport and unable to transport monosaccharides (Jung et al. 2015). Recent studies from watermelon (*Citrullus lanatus*; Ren et al. 2018) or muskmelon (*Cucumis melo*; Cheng et al. 2018) also indicate a role for TST homologs in catalyzing sucrose import

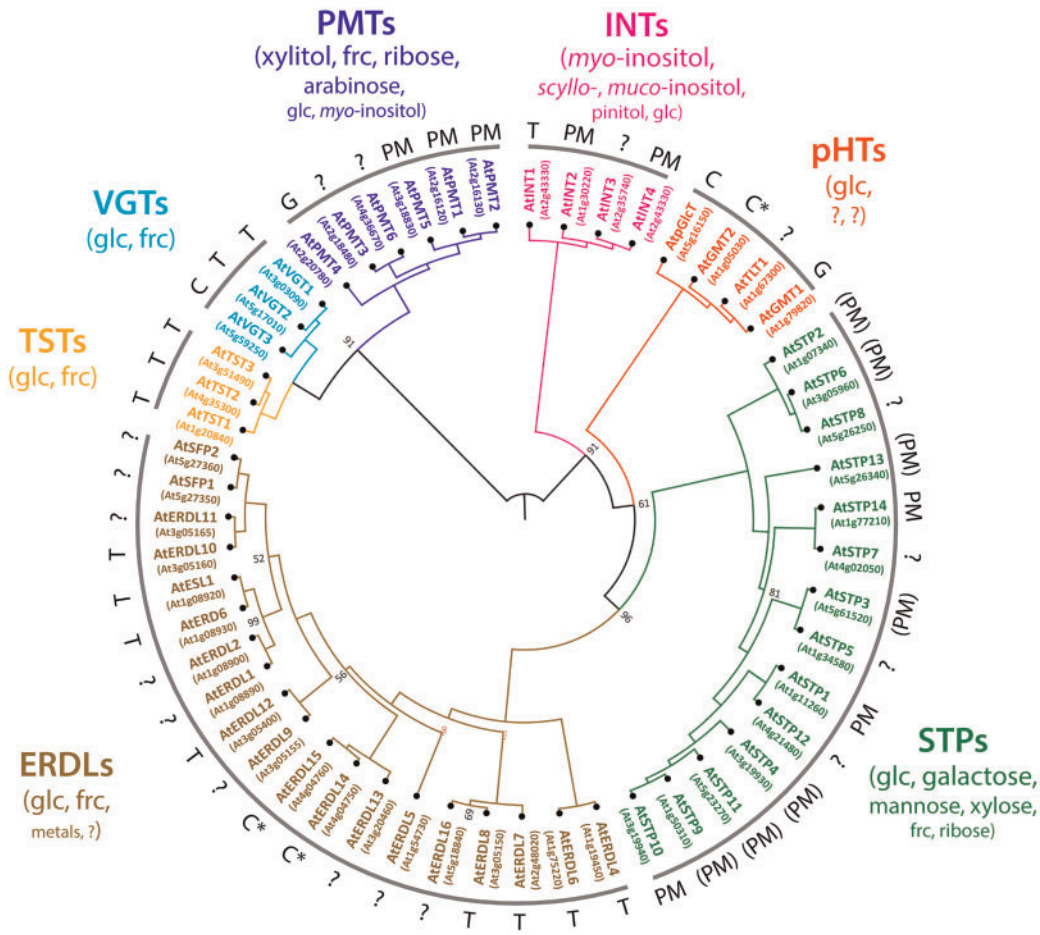

Fig. 2 Phylogenetic tree of the 53 Arabidopsis MST family proteins based on their nucleotide sequences. Numbers at nodes show node support values. Only values <100 are shown. MST subfamilies are abbreviated as follows: PMTs, POLYOL MONOSACCHARIDE TRANSPORTERs; VGTs, VACUOLAR GLUCOSE TRANSPORTERs; TSTs, TONOPLAST SUGAR TRANSPORTERs; ERDLs, EARLY-RESPONSE TO DEHYDRATION SIX-LIKE proteins; STPs, SUGAR TRANSPORTERs; pHTs, PLASTIDIC HEXOSE TRANSPORTERs; INTs, INOSITOL TRANSPORTERs. Names of individual transport proteins are given together with their Arabidopsis identifier (in parentheses). Letters outside of the circle indicate the subcellular localization of the proteins: PM, plasma membrane; T, tonoplast; C, chloroplast; G, Golgi apparatus. Asterisks indicate predicted chloroplast localization due to identification of chloroplast transit peptide sequences (ChloroP 1.1; Emanuelsson etal. 1999). Letters in parentheses indicate presumptive localization, e.g. when localization has been shown in heterologous systems. Question marks indicate unknown subcellular localization. Tested and putative transport substrates are listed for each subfamily. A larger font size indicates higher affinity of subfamily members for substrate. The MST nucleotide sequences were obtained from the aramemnon database (Schwacke etal. 2003). Multiple sequence alignment for all MST sequences was built using Clustal Omega (Sievers etal. 2011). Bayesian phylogenetic analysis was performed with MrBayes version 3.2.6 (Ronquist and Huelsenbeck 2003) using the HKY + I + G model. Mr Bayes was run by conducting two parallel Metropolis-coupled Monte Carlo Markov chain analyses with four chains for 2 million generations. The SD of split frequencies was <0.01. The tree file was visualized using FigTree v.1.4.3.

into the vacuoles of sucrose-storing melon fruit cells. Thus, sucrose transport by TST proteins may be restricted to plants with such specialized sucrose-storing or -utilizing sink tissues. In other words, it is still unknown how sucrose enters the vacuole of higher plant mesophyll cells.

Having discussed how glucose, fructose and sucrose might enter the plant vacuole, it is also important to have a look at proteins catalyzing the export of the corresponding solutes. Export of sucrose, glucose and fructose from vacuoles into the cytosol is catalyzed by a variety of sugar porters, namely the proton-coupled sucrose exporter SUCROSE TRANSPORTER 4 (SUT4) (Schneider et al. 2012), the proton-coupled glucose exporter EARLY RESPONSIVE TO DEHYDRATION 6 (six)-LIKE 6 (ERDL6) (Poschet et al. 2011, Klemens et al. 2014), the glucose facilitator EARLY-RESPONSIVE TO DEHYDRATION SIX-LIKE 1 (ESL1) (Yamada et al. 2010) and the SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERs isoforms (SWEET) SWEET2, 16 and 17 in Arabidopsis (Chardon et al. 2013, Klemens et al. 2013, Guo et al. 2014, Chen et al. 2015). All of these carriers reside in the vacuolar membrane, but some of their genes show major expression in leaf vein parenchyma cells (Yamada et al. 2010, Chardon et al. 2013) while others are expressed in almost all plant cells (Poschet et al. 2011). It will be a great challenge to

dissect the exact cellular functions of all vacuolar-located sugar carriers in more detail.

The Right Sugar Has to Be at the Right Cellular Site to Confer Stress Tolerance

One obvious problem in understanding the function of sugars in stress tolerance is that one has to know which sugar is at what concentration and at what time at a specific cellular location. Moreover, optimized experimental protocols spanning various levels of analyses have to be established to clarify the exact functions of selected sugars in stress tolerance. That the use of mutants and in-depth analyses are of critical importance for our understanding of, for example, low temperature tolerance of Arabidopsis can be impressively illustrated by demonstrating the function of raffinose in frost tolerance.

Raffinose is known to be of comparatively low abundance in plants grown at standard temperatures but increases markedly in concentration upon onset of low temperatures (Jänkänpää et al. 2012, Nägele and Heyer 2013). An Arabidopsis mutant lacking raffinose biosynthesis is virtually free of this sugar and does not show altered electrolyte release properties, which first led to the conclusion that raffinose is not as important for frost protection (Zuther et al. 2004) as previously proposed (Nishizawa et al. 2008). However, a more detailed analysis of chloroplast functions in cold-treated raffinose-free mutants revealed that photosynthetic parameters such as maximum quantum yield of PSII were strongly impaired in these plants (Knaupp et al. 2011), pointing to an important function of this sugar in protecting thylakoid processes rather than serving extrachloroplastic functions.

To modify the sugar compartmentation substantially it seems attractive to alter the activities of vacuolar sugar transporters since the vacuole is by far the biggest cellular storage organ for most of the sugar present in higher plants. By exploiting different Arabidopsis mutants exhibiting either increased or decreased activities of the corresponding transport proteins, it was indeed possible to modify the subcellular distribution of the three most abundant sugars, glucose, fructose and sucrose, in low temperature-challenged leaves.

Overexpression of the *ERDL6* homolog from sugar beet, *B. vulgaris* INTEGRAL MEMBRANE PROTEIN (*Bv*IMP) (Poschet et al. 2011)—originally named PCR CLONE 4 (PCR4) (Chiou and Bush 1996)—in Arabidopsis led to a lower glucose concentration in leaves upon onset of low temperatures, alongside unaltered fructose and sucrose levels, as compared with corresponding changes in wild-type plants (Klemens et al. 2014). These changes are not surprising, given that *Bv*IMP is a glucose-specific, proton-driven sugar exporter as revealed by electrophysiological studies (Klemens et al. 2014). Importantly, these *BvIMP* overexpressor plants exhibit a markedly reduced frost tolerance (Klemens et al. 2014), indicating that glucose accumulation in leaf mesophyll vacuoles is more important for frost tolerance than sucrose accumulation.

A further dissection of the individual functions of glucose and fructose for frost tolerance was possible by analyzing Arabidopsis mutants with altered activities of the vacuolar proteins SWEET16 and SWEET17. SWEET17 is a fructose-specific transporter facilitating export of this monosaccharide into the cytosol (Chardon et al. 2013), while SWEET16 exerted high activity for facilitating glucose exchange but low activity for sucrose and fructose exchange across the vacuolar membrane (Klemens et al. 2013). Interestingly, SWEET17 loss-of-function plants exhibit drastically increased fructose levels in leaves but frost tolerance was not altered (Chardon et al. 2013). In contrast, overexpression of *SWEET16* causes only minor changes in fructose and sucrose levels, but led to significantly higher glucose levels than in the wild type (Klemens et al. 2013).

In summary, these independent analyses support the notion that vacuolar glucose levels are of critical importance for frost tolerance in Arabidopsis. The finding that glucose has to accumulate in the vacuolar lumen and not outside of this organelle is underlined by the detailed examination of frost tolerance properties of Arabidopsis mutants lacking the vacuolar monosaccharide importers TST1 and TST2 (Wormit et al. 2006). These mutants show strongly reduced total levels of monosaccharides upon onset of low temperatures since the vacuolar compartment is not accessible (Wormit et al. 2006). However, cytosolic glucose levels in these mutants are higher than in wild-type plants (Wingenter et al. 2010), but frost tolerance is impaired (Klemens et al. 2014).

Fructans as Sugar Polymers with Pre-Destined Protective Properties Against Cold Stress

Damage to cellular membranes during cold and freezing temperatures needs to be prevented at all costs. Plasma membrane bursting occurs post-freezing, by thawing-induced water influx into the cytosol and vacuole when non-cold-adapted, shrunken cells exhibit highly negative water potentials. Bursting of the vacuolar membrane due to cold-induced attack of ROS can, however, occur pre-freezing and then cause severe damage to the whole cell, leading to apoptosis following massive acidification (due to the release of protons) and flooding of the cytosol with catalytic enzymes, metabolic degradation products and potentially hazardous compounds that had been sequestered into the vacuole for detoxification. In addition to the aforementioned beneficial accumulation of monosaccharides and sucrose under cold stress, higher molecular sugars and sugar polymers such as fructans act as direct protectors of vacuolar lipid bilayers under stress conditions (Valluru et al. 2008).

Structurally, fructans represent sucrose-derived polymers of fructose and exhibit a remarkable structural variability due to the specific action of various enzyme activities, linking the fructose moieties to different positions within a fructan core molecule. Accordingly, the linkage position of the fructose residues determines the type of fructan. This linkage normally occurs at one of the two primary hydroxyl residues (OH-1 or OH-6), leading to the formation of two basic types of simple fructan, namely inulin-type fructans, where the fructosyl residues are linked by β-2,1-linkages, and levan-type fructans where the fructosyl residues are linked by β-2,6-linkages. In any case, fructan metabolism is highly

regulated by both sugar availability and subcellular distribution. Obviously, biosynthesis of fructans depends on the loading of sucrose building blocks from the cytosol into the vacuole since the enzymes catalyzing fructan polymerization, SUCROSE:SUCROSE FRUCTOSYLTRANSFERASE (1-SST) and FRUCTAN:FRUCTAN FRUCTOSYLTRANSFERASE (1-FFT), are located at the luminal side of this organelle (**Fig. 1**; Darwen and John 1989, Van den Ende 1995). Proteins catalyzing sucrose import into fructan-synthesizing vacuoles have not been characterized yet, but the identification of the vacuolar sucrose loader of sugar beet as a TST family homolog (BvTST2;1; Jung et al. 2015) clearly suggests a function of TST proteins also in the sucrose loading of vacuoles of, for example, chicory (*Cichorium intybus*) or Jerusalem artichoke (*Helianthus tuberosus*). Possible cold-induced phosphorylation and, therefore, activation of TST transport proteins (Wingenter et al. 2011, Schulze et al. 2012) could then secondarily trigger the activity of fructan biosynthesis enzymes since high vacuolar monosaccharide and sucrose concentrations can drive fructan biosynthesis by acting as inhibitors of fructan breakdown enzymes (Lothier et al. 2007, Lothier et al. 2014). Additionally, high cytosolic sugar thresholds promote fructan biosynthesis by activating the expression of the *1-SST* and *1-FFT* genes (Lu et al. 2002, Maleux and Van den Ende 2007).

For a long time, fructans have been linked to plant survival during prolonged periods of stress, e.g. during dehydration (Pontis 1989, De Roover et al. 2000). In cereals, fructan accumulation can channel growth-related water fluxes and reduce the otherwise osmotic problems of excess amounts of sucrose, an alternative storage sugar. In developing barley grains, fructans have been identified in the extracellular cavity sap and they possibly contribute there to membrane protection of the embryonic tissue during maturation and desiccation of the developing cereal grain (Peukert et al. 2014). They also play essential roles in frost tolerance of winter-annual graminaceaous and fructan-synthesizing dicots, and inulin-type fructans in particular have been shown to increase membrane stability during frost temperatures (Hincha et al. 2007).

Vacuolar-based fructans have strong antioxidant properties similar to polyphenols, and their hydroxyl radical (·OH)-scavenging potency exceeds that of sucrose and the monosaccharides glucose and fructose. In vitro ROS quenching experiments with different sugars and fructan types clearly demonstrated different quenching efficiencies of the individual sugar species, with inulin having the highest ·OH-scavenging efficiency of the tested substrates (Peshev et al. 2013), and suggest that the composition of the vacuolar and apoplastic fructan cocktails most probably influences the ROS-scavenging efficiency of stressed plants. ·OH scavenging by vacuolar fructans possibly leads to the splitting of fructans and formation of ·OH-dependent polymerization products between fructan-split products and vacuolar-based metabolites (Matros et al. 2015). Such products as well as the resulting fructan polymers with a higher degree of polymerization (DP) may then be subjected to degradation by the action of vacuolar or cell wall-bound cold-induced FRUCTAN 1-EXOHYDROLASEs (1-FEHs) (Michiels et al. 2004, del Viso et al. 2009, Asega et al. 2011). The discovery of these hydrolytic enzymes in the apoplast also indicated the occurrence of extracellular fructans, and their accumulation there has also been linked to response to drought and cold conditions.

Export of fructans from the vacuole and out of the cytosol is most probably transporter dependent and delicately regulated. The plethora and apparent redundancy of the different vacuolar sugar transport protein families (**Fig. 2**) suggest a neofunctionalization for some of these proteins also for the export of fructans or fructan adducts in fructan-synthesizing species and underlines the existence of and need for regulation mechanisms. It remains to be analyzed whether tonoplast-localized MST family members, e.g. ERDL transporters, or tonoplast and plasma membrane-localized SWEET proteins have a role in the export of fructans under these conditions. In addition, concomitant sucrose efflux mediated by vacuolar sucrose transporters (SUT4-type; **Fig. 1**) could also contribute to the fine-tuning of fructan biosynthesis and drive synthesis of high DP fructans by shifting 1-FFT-mediated polymerization towards high DP donor molecules.

Since fructans combine high water solubility with high affinity for and interaction with membranes (Vereyken et al. 2001, Hincha et al. 2007, Peshev et al. 2013), it seems quite likely that accumulation of high molar vacuolar fructans greatly contributes to the frost tolerance of inulin-storing tissues such as the tap roots of chicory. The superior antioxidant behavior and membrane protective function of inulin-type fructans may also be why the otherwise morphological and anatomically similar storage roots of chicory and the sucrose-only storing tap roots of sugar beet (*B. vulgaris*) exhibit contrasting tolerance towards freezing temperatures (chicory: Van den Ende et al. 1995, Skinner and Gustine 2002, sugar beet: Barbier et al. 1982).

Biotechnological adjustment of the activity of transport proteins involved in fructan biosynthesis seems, therefore, relevant for the generation of crops with enhanced tolerance against ·OH-producing stress stimuli. Genetically modified plants with the ability to integrate fructan composition and vacuolar sugar transport into stress response pathways could then benefit from a fructan profile adjusted and required for adequate responses to specific stresses.

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The authors have no conflicts of interest to declare.

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