the plant journal

SEB Society for Experimental Biology

The Plant Journal (2014) **79,** 544–567 doi: 10.1111/tpj.12509

SMALL MOLECULES: FROM STRUCTURAL DIVERSITY TO SIGNALLING AND REGULATORY ROLES

Trehalose metabolism in plants

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Received 9 January 2014; revised 18 February 2014; accepted 3 March 2014; published online 19 March 2014.

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SUMMARY

Trehalose is a quantitatively important compatible solute and stress protectant in many organisms, including green algae and primitive plants. These functions have largely been replaced by sucrose in vascular plants, and trehalose metabolism has taken on new roles. Trehalose is a potential signal metabolite in plant interactions with pathogenic or symbiotic micro-organisms and herbivorous insects. It is also implicated in responses to cold and salinity, and in regulation of stomatal conductance and water-use efficiency. In plants, as in other eukaryotes and many prokaryotes, trehalose is synthesized via a phosphorylated intermediate, trehalose 6-phosphate (Tre6P). A meta-analysis revealed that the levels of Tre6P change in parallel with sucrose, which is the major product of photosynthesis and the main transport sugar in plants. We propose the existence of a bi-directional network, in which Tre6P is a signal of sucrose availability and acts to maintain sucrose concentrations within an appropriate range. Tre6P influences the relative amounts of sucrose and starch that accumulate in leaves during the day, and regulates the rate of starch degradation at night to match the demand for sucrose. Mutants in Tre6P metabolism have highly pleiotropic phenotypes, showing defects in embryogenesis, leaf growth, flowering, inflorescence branching and seed set. It has been proposed that Tre6P influences plant growth and development via inhibition of the SNF1-related protein kinase (SnRK1). However, current models conflict with some experimental data, and do not completely explain the pleiotropic phenotypes exhibited by mutants in Tre6P metabolism. Additional explanations for the diverse effects of alterations in Tre6P metabolism are discussed.

Keywords: abiotic stress, plant–microbe interactions, sucrose, trehalose, trehalose 6-phosphate, starch.

INTRODUCTION

Last year marked the centenary of a paper reporting the detection of trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) in *Selaginella lepidophylla*, the first record of the occurrence of trehalose in plants (Anselmino and Gilg, 1913). Trehalose was subsequently found in green algae, mosses, liverworts and ferns, but, apart from a small number of desiccation-tolerant resurrection plants, there were few reports of trehalose being present in angiosperms (Elbein, 1974; Kandler and Hopf, 1980; Drennan *et al.*, 1993; Iturriaga *et al.*, 2000). The amounts of trehalose found in flowering plants were generally very low, and were often suspected to have a fungal or microbial origin (Kandler and Hopf, 1980). With little evidence to the contrary, there was a general consensus that trehalose metabolism was unimportant in most flowering plants, or even absent.

This view was challenged by the finding of families of genes encoding active trehalose phosphate synthase (TPS; EC 2.4.1.15) and trehalose phosphatases (TPP; EC 3.1.3.12) in *Arabidopsis thaliana* (Blázquez *et al.*, 1998; Vogel *et al.*, 1998; Leyman *et al.*, 2001), establishing beyond doubt that this species has the capacity to synthesize trehalose via the phosphorylated intermediate trehalose 6-phosphate (Tre6P). In parallel, attempts to engineer trehalose production by introduction of heterologous fungal or bacterial enzymes gave rise to transgenic plants with a wide range of developmental anomalies (Goddijn *et al.*, 1997;

Pilon-Smits *et al.*, 1998; Goddijn and van Dun, 1999), providing evidence that perturbation of trehalose metabolism has far-reaching effects on plant metabolism and development. Together, these observations overturned the previous consensus; instead of being unimportant and of little interest, trehalose metabolism is now recognized to play an essential and pervasive role in the life of plants.

Trehalose is highly soluble but chemically unreactive due to its non-reducing nature, making it compatible with cellular metabolism even at high concentrations. It is widespread in bacteria, fungi and invertebrates, which use it as an osmolyte and stress protectant, as well as for carbon storage and transport (Elbein, 1974; Benaroudj *et al.*, 2001; Bonini *et al.*, 2004). In vascular plants, many of these functions are performed by a different non-reducing disaccharide – sucrose (Lunn, 2008). Typically, higher plants have 100–1000 times more sucrose than trehalose (Carillo *et al.*, 2013), and the flux of newly fixed carbon into sucrose is approximately four orders of magnitude greater than the flux into trehalose in *A. thaliana* rosettes (Szecowka *et al.*, 2013). The reasons for the primacy of sucrose in plant metabolism are uncertain, but it has been suggested that the lower viscosity of concentrated sucrose solutions made this sugar more suitable than trehalose for transport in the narrow sieve elements of the phloem (MacRae and Lunn, 2012).

In this review, we discuss the functions of trehalose metabolism in plants. Research in the last 10 years has revealed a previously unsuspected role for Tre6P in the regulation of many aspects of plant metabolism and development (Schluepmann *et al.*, 2003). We discuss the intimate relationship between Tre6P and sucrose metabolism in plants (Lunn *et al.*, 2006), and highlight recent advances in understanding the functions of Tre6P and how it exerts such a profound influence in the lives of plants. We also consider the potential role of trehalose itself as a signal metabolite in abiotic stress responses and in interactions of plants with other organisms, including fungal and bacterial pathogens, insects, parasitic plants, mycorrhizae, rhizobia and non-symbiotic rhizobacteria (Fernandez *et al.*, 2010).

ENGINEERING TREHALOSE METABOLISM IN PLANTS

In addition to its well-known stress-protecting properties in micro-organisms and some resurrection plants, trehalose is a valuable commodity with applications in the pharmaceutical and food industries. These considerations motivated early attempts to engineer trehalose biosynthesis in tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) by introducing bacterial or fungal genes encoding trehalose-synthesizing enzymes (Romero *et al.*, 1997; Goddijn and van Dun, 1999). Although the heterologous *TPS* genes were strongly expressed, there was either no change or only a small increase in the amount of trehalose in the plants (Romero *et al.*, 1997; Goddijn and van Dun, 1999). Blocking the activity of the endogenous plant trehalase by validamycin A resulted in a clear increase in trehalose levels, supporting the idea that high trehalase activity prevents accumulation of trehalose in higher plants (Goddijn and van Dun, 1999).

Despite there being little or no change in trehalose levels, the transgenic plants showed improved stress tolerance, and, unexpectedly, a wide array of phenotypic abnormalities (summarized in Table 1). Tobacco plants over-expressing *TPS* exhibited stunted growth, developing small, dark, lancet-shaped leaves with increased photosynthetic capacity and delayed senescence (Goddijn *et al.*, 1997; Pilon-Smits *et al.*, 1998; Goddijn and van Dun, 1999; Pellny *et al.*, 2004), while constitutive *TPS* expression in *A. thaliana* led to stunted growth, early flowering and increased shoot branching (Schluepmann *et al.*, 2003). A key finding was that broadly opposite developmental phenotypes were obtained when genes encoding bacterial or yeast TPP enzymes were over-expressed in *A. thaliana* or tobacco (Schluepmann *et al.*, 2003; Paul *et al.*, 2008). Although the transgenic plants again exhibited improved stress tolerance, their leaves were slightly bigger and paler, and flowering and senescence were delayed. The obvious conclusion from these experiments is that changes in the level of Tre6P were responsible for the opposite developmental phenotypes resulting from TPS versus TPP over-expression (Schluepmann *et al.*, 2003). It is worth noting that the phenotypes may be generated not only by changes in Tre6P levels *per se*, but also via resulting changes in the downstream metabolism of Tre6P by endogenous TPP activities.

In conjunction with the discovery that *A. thaliana* possesses enzymes for trehalose synthesis (Blázquez *et al.*, 1998; Vogel *et al.*, 1998), the highly pleiotropic phenotypes of transgenic plants expressing heterologous *TPS* and *TPP* genes led to recognition of the importance of trehalose metabolism in plants. This was reinforced by transcriptomic studies that revealed prominent changes in expression of endogenous plant *TPS*, *TPP* and trehalase (*TRE*) genes in response to various abiotic stresses (Seki *et al.*, 2002; Kaplan *et al.*, 2004; Thimm *et al.*, 2004; Iordachescu and Imai, 2008; Nakashima *et al.*, 2009). These findings established priorities for research into trehalose metabolism in plants: to measure the levels of Tre6P and trehalose, to learn when their levels change and how they are regulated, to understand the biological activities of these metabolites in plant metabolism, development and growth, to understand how trehalose metabolism contributes to stress resistance, and to develop strategies to engineer trehalose metabolism in a more specific manner to improve plant growth and stress tolerance.

ORIGIN, EVOLUTION AND FUNCTION OF TREHALOSE METABOLIC ENZYMES IN PLANTS

There are several pathways of trehalose biosynthesis in bacteria and archaea, but probably the most common

Table 1 Metabolic and developmental phenotypes in mutants and transgenic plants with altered levels of Tre6P

	Impact of altered Tre6P	References
A. thaliana (embryogenesis): decreased Tre6P		
Embryo size	Decreased	1, 2
Chloroplast development	Normal	1
Developmental program	Normal	1
Cell division	Decreased	1
Protein content	Decreased	1
Storage lipids	Decreased	1
Cell wall	Thickened	1
Starch	Increased	1
Sugars	Increased	1
A. thaliana (seedlings or rosettes): increased Tre6P		
Germination on high exogenous sugar	Increased tolerance[a]	2, 12
Germination on high ABA	Increased tolerance[a]	12
Hypocotyl	Thick, stunted	3
Leaf area	Decreased[b]	2, 3, 4
Specific leaf area (m^2 g^{-1})	Decreased	5
Dry matter content	Increased	5
Cell size	Decreased	13
Cell number per leaf	Decreased[c]	13
Chlorophyll	Increased	2
Photosynthesis	Increased	4
Anthocyanin content	Increased	2, 3, 6
Starch content	Increased	2, 4
Starch accumulation rate	Marginally increased	7
Starch degradation rate	Inhibited	5, 7
Sucrose content	Decreased	5,7
Glc6P	Decreased	2, 7
Glc1P	Decreased	7
Flowering time	Early[d]	2,4, 8, 9
Floral stem	More branching	2
Seed development	Many abortions	2
Leaf senescence	Earlier	6
Stomata	Larger aperture	10
S. tuberosum (tubers): decreased Tre6P		
Shape	Modified	11
Size	Decreased[e]	11
Lenticel number	Increased	11
Sprouting	Delayed[f]	11
Starch content	Decreased	11
Sucrose content	Decreased	11
Glc6P, Glc1P, UDPglucose	Decreased	11
Respiration	Increased	11
Flux distribution to starch and sucrose	Unchanged	11

The responses to decreased Tre6P levels in *A. thaliana* embryos (*tps1* knockout lines) and increased or decreased Tre6P levels in *A. thaliana* seedlings and rosettes (mainly *35S::otsA* and *35S::otsB* lines, and inducible *otsA* lines) and *Solanum tuberosum* tubers (*B33::otsB* lines) are summarized. In *A. thaliana* rosettes, the listed phenotypes reflect the response to an increase in Tre6P above wild-type levels; most show reciprocal changes in *35S::otsB* lines, although it should be noted that these show no or only a marginal decrease in Tre6P. In potato tubers, the changes represent the difference between *B33::otsB* lines and wild-type tubers; *B33::otsA* tubers showed only a small increase in Tre6P (Debast *et al.*, 2011).

References: (1) Gómez *et al.* (2006); (2) Schluepmann *et al.* (2003); (3) Paul *et al.* (2010); (4) Goddijn and van Dun (1999); (5) Yadav *et al.* (2014); (6) Wingler *et al.* (2012); (7) Martins *et al.* (2013); (8) Avonce *et al.* (2004); (9) Wahl *et al.* (2013); (10) Gómez *et al.* (2010); (11) Debast *et al.* (2011); (12) Avonce *et al.* (2004); (13) Ivakov (2011).

[a]The study described in reference 13 used AtTPS1 and separated these germination phenotypes from developmental phenotypes in older plants.

[b]Decreased leaf area is a poor indicator of a change in growth rate; it is least partly due to changes in leaf composition, including a higher specific leaf area (i.e. less area per unit FW) and a higher dry weight content per unit FW (Yadav *et al.*, 2014). Note also that biomass is not increased in *35S::otsB* lines compared to wild-type plants; instead, the increased size of older rosettes of *35S::otsB* lines is mainly due to a delay in flowering (Yadav *et al.*, 2014).

[c]Cell number per leaf was also decreased in *35S::otsB* lines.

[d]High Tre6P promotes the *CO*/*FT* photoperiod pathway and miR156/*SPL3* maturity pathway (Wahl *et al.*, 2013). This study used rescued *tps1* lines and *amiR-TPS1* lines as well as cell-specific over-expression of *AtTPS1*.

[e]Size was decreased in both *B33::otsB* and *B33::otsA* tubers.

[f]Possibly via inhibition of ABA catabolism (Debast *et al.*, 2011).

pathway, and the only one present in eukaryotes, is the two-step pathway catalysed by TPS and TPP via Tre6P (Cabib and Leloir, 1958; Avonce *et al.*, 2010). Following initial identification of the *AtTPS1*, *TPPA* and *TPPB* genes in *A. thaliana* (Blázquez *et al.*, 1998; Vogel *et al.*, 1998), genome sequencing revealed that there are 11 genes encoding TPS or TPS-like proteins (*AtTPS1*–*AtTPS11*) and ten genes encoding TPP (*AtTPPA*–*AtTPPJ*) in the *A. thaliana* genome, but only a single identifiable trehalase-encoding gene (*AtTRE1*; Arabidopsis Genome Initiative, 2000; Leyman *et al.*, 2001). Surveys of other plant genome sequences and expressed sequence tags (ESTs) revealed the presence of *TPS* and *TPP* genes in all major plant taxa, including both monocotyledonous and eudicotyledonous angiosperms (Avonce *et al.*, 2006, 2010; Lunn, 2007). Based on the widespread occurrence of *TPS* and *TPP* genes, it has been suggested that the capacity to synthesize trehalose is universal in the plant kingdom (Lunn, 2007).

Phylogenetic analysis of the *A. thaliana TPS* genes showed that they cluster into two distinct sub-families, designated class I (*AtTPS1*–*4*) and class II (*AtTPS5*–*11*), which probably originated from a common ancestor (Leyman *et al.*, 2001; Avonce *et al.*, 2010; Yang *et al.*, 2012). These two clades are present in all major plant taxa, including chlorophyte green algae, indicating that they diverged early in evolution of the green plant lineage (Lunn, 2007). There is clear evidence of independent *TPS* and *TPP* gene duplications within various plant lineages (Lunn, 2007; Ramon *et al.*, 2009; Vandesteene *et al.*, 2010, 2012). Both class I and class II TPS proteins contain a glycosyltransferase domain similar to the TPS enzymes from yeast (*Saccharomyces cerevisiae*; *Sc*TPS1) and *Escherichia coli* (otsA; Figure 1). However, only class I isoforms have been shown to have TPS activity *in vitro* and to

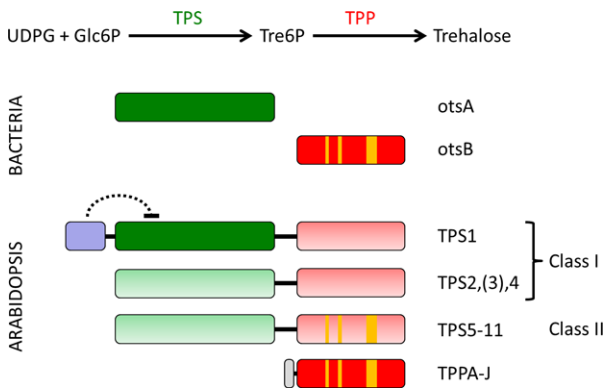

Figure 1. Domain architecture of bacterial and *A. thaliana* TPS and TPP proteins.
The glycosyltransferase domain of TPS proteins is shown in green. The phosphatase domain of TPS and TPP proteins is shown in red, with yellow bars representing three active-site motifs that are characteristic of haloacid dehalogenase family enzymes. Solid shading indicates that the corresponding catalytic activity has been demonstrated *in vitro*. The AtTPS1 protein has an N-terminal domain (purple) whose removal activates the enzyme *in vitro*. The other class I TPS proteins lack this auto-inhibitory domain. *AtTPS3* is thought to be a pseudogene and is not expressed.

reproducibly complement the yeast *tps1Δ* mutant, which cannot grow on glucose-containing medium (Blázquez *et al.*, 1998; Zentella *et al.*, 1999; Vogel *et al.*, 2001; Van Dijck *et al.*, 2002; Valenzuela-Soto *et al.*, 2004; Harthill *et al.*, 2006; Ramon *et al.*, 2009; Vandesteene *et al.*, 2012).

The *A. thaliana* and *S. lepidophylla* TPS1 proteins have an N-terminal domain whose removal increases catalytic activity (Van Dijck *et al.*, 2002). This auto-inhibitory action is linked to an Arg/Leu-rich region within the N-terminal domain. Similar N-terminal extensions containing the auto-inhibitory motif are present in orthologues throughout the plant kingdom, including both the chlorophyte green algal and streptophyte lineages (Lunn, 2007). The N-terminal domain in OtTPS1 from the pico-alga *Ostreococcus tauri* was experimentally confirmed to be auto-inhibitory (Avonce *et al.*, 2010). The presence of this domain in single-celled green algae indicates that it arose early in plant evolution, and its conservation throughout the plant kingdom suggests that it has an important but not yet clearly defined purpose.

Arabidopsis thaliana is unusual among diploid angiosperms in having multiple class I *TPS* genes (*AtTPS1*–*AtTPS4*). *AtTPS3* is thought to be a pseudogene because the coding region for the glycosyltransferase domain appears to be corrupted and *AtTPS3* transcripts are not reproducibly detectable by quantitative RT-PCR (Lunn, 2007; Vandesteene *et al.*, 2010; Yadav *et al.*, 2014). In contrast to AtTPS1, the AtTPS2–AtTPS4 isoforms lack the N-terminal auto-inhibitory domain (Lunn, 2007). AtTPS2–AtTPS4 did not complement the yeast *tps1Δ* mutant (Vandesteene *et al.*, 2010). However, it has been noted that TPS active-site residues (Gibson *et al.*, 2004) are highly conserved in these isoforms (Lunn, 2007; Vandesteene *et al.*, 2012), suggesting that they do have potential to be catalytically active under some circumstances. Genes encoding similarly truncated class I TPS isoforms are present in *Arabidopsis lyrata*, *Brassica rapa* and other species in the Brassicaceae (Lunn, 2007), but not in papaya (*Carica papaya*), which belongs to the Caricaceae, a sister family to the Brassicaceae within the order Brassicales (Ming *et al.*, 2008). The function of the truncated class I TPS isoforms in the Brassicaceae is unknown, but, in *A. thaliana*, the *AtTPS2* and *AtTPS4* genes are expressed predominantly in developing seeds/siliques (Schmid *et al.*, 2005), suggesting a particular role in reproductive organs.

In addition to the glycosyltransferase domain, class I and class II TPS proteins have a C-terminal region that resembles the phosphatase domain of yeast TPP (encoded by the *ScTPS2* gene; Bell *et al.*, 1998; Leyman *et al.*, 2001). The TPP-like domain of the class II TPSs includes three amino acid motifs that are characteristic of the active sites of enzymes from the L-2-haloacid dehalogenase superfamily, with the initial Asp residue in motif I [DX(D/T/Y)X(T/V)(L/V/I)] acting as a nucleophile to form a phospho-acyl intermediate during catalysis (Rao *et al.*, 2006; Fieulaine *et al.*, 2005). Despite the high conservation of TPP active-site residues, the class II TPS proteins are unable to complement the yeast *tps2Δ* mutant, which lacks TPP activity (Vogel *et al.*, 2001; Harthill *et al.*, 2006; Ramon *et al.*, 2009), and *in vitro* assays of heterologously expressed AtTPS5, AtTPS7 and AtTPS8 revealed no detectable TPP activity (Harthill *et al.*, 2006).

The function of the class II TPS proteins is unknown. In rice (*Oryza sativa*), some of them were found to associate with OsTPS1 in yeast two-hybrid and bimolecular fluorescence complementation assays (Yang *et al.*, 2012). However, direct evidence of TPS1-class II TPS interactions in wild-type plant cells, for example by co-immunoprecipitation, is still lacking.

All 10 isoforms of TPP in *A. thaliana* complement the yeast *tps2Δ* mutant (Vogel *et al.*, 1998; Vandesteene *et al.*, 2012), as do several of their homologues from rice, maize (*Zea mays*) and grapevine (*Vitis vinifera*; Pramanik and Imai, 2005; Satoh-Nagasawa *et al.*, 2006; Fernandez *et al.*, 2012), indicating that they have TPP activity. The RAMOSA3 (RA3) isoform of TPP in maize is of particular interest, because a lesion in the gene encoding this enzyme in the *ra3* mutant increases branching of female and male inflorescences (Satoh-Nagasawa *et al.*, 2006). Inflorescence primordia from the *ra3* mutant have less trehalose and a lower trehalose:Tre6P ratio than wild-type primordia (Carillo *et al.*, 2013). However, it is not yet established whether these metabolic differences contribute to the developmental phenotype, as it remains a possibility that the RA3

protein also has a non-catalytic function, perhaps as a transcriptional regulator (Satoh-Nagasawa *et al.*, 2006).

Little is known about the regulation of TPS and TPP activities in plants. In *A. thaliana*, *AtTPS1* transcripts were detected in most parts of the plant, showing little variation in abundance between organs or at various developmental stages (Schmid *et al.*, 2005). However, expression is not necessarily ubiquitous, as *in situ* hybridization revealed that *AtTPS1* transcripts are localized in specific zones in the shoot apex (Wahl *et al.*, 2013). Analysis of *A. thaliana tps1* null mutants has shown that AtTPS1 is essential for embryogenesis, vegetative growth and flowering (Eastmond *et al.*, 2002; van Dijken *et al.*, 2004; Gómez *et al.*, 2006; Gómez *et al.*, 2010). The *A. thaliana tps1-11*, *tps1-12* and *tps1-13* mutants carrying weak alleles of *AtTPS1* have less Tre6P than wild-type plants (Gómez *et al.*, 2010), as do *35S::amiR-TPS1* plants carrying an artificial micro-RNA suppression construct targeted to *AtTPS1* (Wahl *et al.*, 2013). This suggests that AtTPS1 exerts at least some control over Tre6P levels.

AtTPS1 is potentially phosphorylated on Ser252 by calcium-dependent protein kinases but not by SNF1-related kinase 1 (SnRK1; Glinski and Weckwerth, 2005). The effect of phosphorylation on AtTPS1 activity has not yet been investigated. Although antisense suppression of SnRK1 in developing pea (*Pisum sativum*) embryos increased Tre6P levels, expression of *PsTPS1* and the sucrose content were also higher, so the effect on Tre6P levels may have been an indirect consequence of reducing SnRK1 activity (Radchuk *et al.*, 2009). *S. lepidophylla* TPS1 (SlTPS1) is activated by several cations (K^+, Mg^{2+} and Ca^{2+}) but is insensitive to trehalose or sucrose (Valenzuela-Soto *et al.*, 2004). However, as this desiccation-tolerant resurrection plant is capable of accumulating extremely high levels of trehalose, the kinetic properties of SlTPS1 may not be typical of TPS enzymes from other plants.

Expression of many of the class II *TPS* and *TPP* genes is restricted to specific cell types, often in meristematic regions, and dependent on the plant's developmental stage (Schmid *et al.*, 2005; Ramon *et al.*, 2009; Vandesteene *et al.*, 2010, 2012). Several show pronounced diurnal rhythms of expression in rosettes, driven by changes in light, sugar content and the circadian clock (Usadel *et al.*, 2008). *AtTPS5* expression is suppressed by carbon starvation and induced by sugars in seedlings and rosettes, whereas expression of *AtTPS8*–*AtTPS11* is induced by carbon starvation and strongly repressed by sugars (Price *et al.*, 2004; Bläsing *et al.*, 2005; Osuna *et al.*, 2007; Yadav *et al.*, 2014). Expression of *AtTPS9*, *AtTPS10*, *ATTPA*, *AtTPPB* and *AtTPPJ* is repressed by nitrogen starvation and/or induced by nitrate (Wang *et al.*, 2003; Scheible *et al.*, 2004). The AtTPS5–AtTPS7 (Moorhead *et al.*, 1999; Harthill *et al.*, 2006) and AtTPS8–AtTPS11 proteins (Glinski and Weckwerth, 2005) are potential targets for phosphorylation by SnRK1 and/or calcium-dependent protein kinases. Sugars promote phosphorylation of AtTPS5–AtTPS7, possibly protecting these proteins, by binding of 14-3-3 proteins, from degradation via the ubiquitin–26S proteasome pathway (Moorhead *et al.*, 1999; Cotelle *et al.*, 2000; Harthill *et al.*, 2006). As the function of the class II TPS isoforms is unknown, it is unclear whether transcriptional and post-translational regulation of their expression and activity affects the levels of Tre6P and trehalose, or signalling by these metabolites.

Apart from evidence that SlTPS1 is located in the cytosol (Van Dijck *et al.*, 2002), little is known about the subcellular compartmentation of TPS and TPP proteins in plants. The *A. thaliana* TPPs are predicted to be located in the cytosol or plastids (Vandesteene *et al.*, 2012; Tanz *et al.*, 2013). Non-aqueous fractionation of *A. thaliana* leaves showed that Tre6P is predominantly located in the cytosol, with estimated *in vivo* concentrations of 4–7 μM in the cytosol, 0.2–0.5 μM in the chloroplasts and 0.05 μM in the vacuole (Martins *et al.*, 2013). Due to the technical limitations of the non-aqueous fractionation method, it was argued that the estimates for the chloroplasts and vacuole should be regarded as upper limits of the *in vivo* concentrations in these organelles, and that the true concentrations may be much lower, or even zero.

The *A. thaliana tre1-1* null mutant has no detectable trehalase activity (van Houtte *et al.*, 2013), indicating that AtTRE1 is the only functional trehalase in this species. A trehalose-hydrolysing activity with an acidic pH optimum has been reported in extracts from some legumes, but the activity was not specific for trehalose and was ascribed to a non-specific α-glucosidase (García *et al.*, 2005; López *et al.*, 2008a,b). AtTRE1 is bound to the plasmalemma, with the active site facing the apoplast (Müller *et al.*, 1995; Frison *et al.*, 2007). The predominantly cytosolic location of Tre6P and the predicted cytosolic location of most of the *A. thaliana* TPPs (see above) imply that trehalose is produced within the cell at a location where it is inaccessible to the apoplastic trehalase. This raises a series of perplexing questions. For example, how does trehalose cross the plasmalemma to become accessible to trehalase, and why is trehalase located in the apoplast? Nothing is known about the transport of trehalose in plants. The potential for apoplastic trehalase to be involved in sensing extracellular trehalose is discussed below in a section on the role of trehalose in plant–microbe interactions.

MEASUREMENT OF TRE6P IN PLANTS

In yeast cells, Tre6P is not only the intermediate for synthesis of the major carbohydrate, but also a regulatory metabolite. It is a competitive inhibitor of glucose phosphorylation by hexokinases (HXK1 and HXK2), affecting the entry of glucose into glycolysis (Blázquez *et al.*, 1993) and the stability of the glycolytic metabolic network (van

Heerden *et al.*, 2014). Plant tissues contain approximately 100 times less Tre6P than yeast cells, making it technically challenging to measure this metabolite reliably in plant extracts. Methods originally developed for yeast research, including a yeast hexokinase inhibition assay (Schluepmann *et al.*, 2003) and HPLC with pulsed amperometric detection (Veyres *et al.*, 2008), lack the necessary sensitivity and specificity, and are especially prone to over-estimation of Tre6P in plant extracts due to interference by other compounds (Lunn *et al.*, 2006).

To overcome these problems, Lunn *et al.* (2006) developed a highly sensitive and specific assay for Tre6P using anion-exchange HPLC coupled to tandem mass spectrometry (LC-MS/MS), which allows as little as 2 fmol of Tre6P to be reliably quantified. The assay is highly specific because it incorporates three sequential filters: (i) baseline separation of Tre6P from other disaccharide monophosphates, (ii) selection of specific parent ions in the first quadrupole, and (iii) selection of specific product ions in the third quadrupole, following optimized fragmentation in the second quadrupole. In addition to Tre6P, at least four peaks, so far unidentified, with the characteristic parent mass of a disaccharide monophosphate are routinely detected in extracts of axenically grown *A. thaliana* seedlings. The specificity of the anion exchange LC-MS/MS assay was demonstrated by showing that the peak assigned to Tre6P was abolished by pre-incubation of plant tissue extracts with purified recombinant *E. coli* TPP (Lunn *et al.*, 2006). LC-MS/MS- or LC-MS-based assays have become the method of choice for measuring Tre6P in plant tissues (Debast *et al.*, 2011; Delatte *et al.*, 2011; Sastre Toraño *et al.*, 2012).

At the time of establishing the assay, Sigma-Aldrich (www.sigma-aldrich.com) was the only source of commercially available Tre6P. Enzymatic analysis of three separate batches of Tre6P supplied by Sigma-Aldrich in 2005–2006 showed that these contained only 60–70% Tre6P (Lunn *et al.*, 2006). Therefore, the concentrations of Tre6P stock solutions were measured enzymatically by two independent methods before use as calibration standards for the LC-MS/MS assay (Lunn *et al.*, 2006). Subsequent mass spectrometric analysis of the Tre6P supplied by Sigma-Aldrich confirmed that this contained <65% Tre6P, with the remainder being a mixture of over 40 compounds including approximately 25 types of fatty acid or fatty acid ester, hexose and pentose phosphates, *myo*-inositol-1,2-cyclic phosphate and 2-hydroxyethyl 12-hydroxyoctadecanoate, an artificial surfactant (see Table S4 in Yadav *et al.*, 2014). From 2011, Sigma-Aldrich offered a new formulation of Tre6P (trehalose 6-phosphate dipotassium salt; catalogue number T4272) with a nominal purity of 95%. Mass spectrometric and enzymatic analysis of a recent batch showed that this product contained no detectable contaminants (Yadav *et al.*, 2014).

The implications of these findings are twofold. First, any measurements of Tre6P prior to 2011 that were based on Tre6P standards supplied by Sigma-Aldrich most likely over-estimated Tre6P levels by up to 67%, unless the standards were independently calibrated as described by Lunn *et al.* (2006). Second, the results of any *in vitro* analysis using Sigma-Aldrich Tre6P prior to 2011 must be interpreted with caution, given the presence of so many contaminants in the Tre6P product available at that time. Although the currently available Sigma-Aldrich stocks of Tre6P have greatly improved purity, it is recommended that this be confirmed for any batch used for *in vitro* experiments, including use as calibration standards in Tre6P assays. For reliable measurement of Tre6P using LC-MS/MS, it is also advisable to spike all samples with an isotopically labelled Tre6P internal standard to allow correction for ion suppression and other matrix effects, and to establish that recoveries of Tre6P during extraction are within an acceptable range (Lunn *et al.*, 2006).

THE TRE6P–SUCROSE NEXUS

Using a rigorously validated LC-MS/MS assay, Lunn *et al.* (2006) found that the level of Tre6P was very low in carbon-starved *A. thaliana* seedlings (18 pmol g^{-1} FW; over 10^3 and 10^4 times lower than glucose 6-phosphate and sucrose, respectively), and increased rapidly when sucrose was supplied exogenously. A strong correlation between Tre6P and sucrose content was also observed in rosettes of soil-grown wild-type *A. thaliana* plants and the starch-deficient *pgm* mutant during the diurnal cycle (Lunn *et al.*, 2006). Subsequent studies have confirmed the correlation between Tre6P and sucrose in *A. thaliana* seedlings (Lunn *et al.*, 2006; Nunes *et al.*, 2013a; Yadav *et al.*, 2014), rosettes (Lunn *et al.*, 2006; Wingler *et al.*, 2012; Carillo *et al.*, 2013; Crumpton-Taylor *et al.*, 2013; Ragel *et al.*, 2013; Sulpice *et al.*, 2014; Yadav *et al.*, 2014), developing seeds (Thiel *et al.*, 2011) and shoot apices (Wahl *et al.*, 2013), as well as in developing potato tubers (Debast *et al.*, 2011) and wheat grains (*Triticum aestivum*; Martínez-Barajas *et al.*, 2013). Strikingly similar ratios of Tre6P to sucrose have been found in independent studies across a wide range of life stages, conditions and species (Table S1). In an extensive study in which nutrient-starved *A. thaliana* seedlings were supplied with a wide range of sugars, sugar analogues, KNO_3, NH_4Cl, K_2SO_4 or KH_2PO_4, Yadav *et al.* (2014) found that Tre6P correlated most strongly with sucrose, and concluded that the response to other sugars and nutrients may be explained via the effect of these treatments on sucrose levels. These results support the conclusion that Tre6P acts as a specific signal of sucrose status, and that other sugars or nutrients that are essential for growth (nitrogen, phosphate and sulfate) either have little influence on Tre6P levels or affect Tre6P indirectly via their effects on sucrose content.

The *in vivo* concentration of Tre6P is determined by the relative rates of Tre6P synthesis by TPS and hydrolysis by TPP. It is unclear whether one or other of these two processes dominates the control of Tre6P levels, or whether both make a significant contribution. To investigate this question, Tre6P and sucrose levels were measured in *A. thaliana* plants engineered to constitutively express the *E. coli* TPS (*35S::otsA*) or the *E. coli* TPP (*35S::otsB*; Yadav *et al.*, 2014). Being heterologous, the bacterial enzymes are likely to be insensitive to the regulatory mechanisms controlling the activities of the endogenous plant TPS and TPP enzymes, potentially breaking the tight linkage between sucrose and Tre6P. The plants were grown under three photoperiods and harvested at the end of the day and the end of the night to obtain material with a wide range of Tre6P and sucrose contents.

The *35S::otsA* plants had elevated levels of Tre6P, as previously observed in similar lines (Schluepmann *et al.*, 2003), but the sucrose content was lower than in wild-type control plants (Yadav *et al.*, 2014). Despite the changes in both metabolites, a strong correlation between Tre6P and sucrose was seen in both day- and night-harvested samples, but with a strong upward shift in the Tre6P:sucrose ratio (Yadav *et al.*, 2014; Figure S1). The Tre6P content of the *35S::otsB* plants was not significantly different from that of wild-type plants, but sucrose levels were increased, particularly under long-day conditions. A strong correlation between Tre6P and sucrose was also observed in these plants, but with a downward shift in the Tre6P:sucrose ratio (Yadav *et al.*, 2014; Figure S1). An induced increase in Tre6P in *A. thaliana* rosettes is also rapidly followed by a small but significant decrease in sucrose in the light and a large decrease in sucrose in the dark (Martins *et al.*, 2013).

These results have two interesting implications (Figure 2). First, the regulation of Tre6P levels by sucrose is so robust that the correlation between Tre6P and sucrose is retained in the presence of heterologous unregulated TPS or TPP activity, albeit with a changed slope. This observation indicates that both synthesis and degradation of Tre6P may be regulated by sucrose. Regulation of the endogenous TPS activities is sufficient to maintain a dependence of Tre6P levels on sucrose content when heterologous TPP activity is introduced, and regulation of endogenous TPP activities is sufficient to maintain a dependence of Tre6P levels on sucrose content when heterologous TPS activity is introduced. Second, the observation that a genetically imposed change in Tre6P leads to a reciprocal change in sucrose indicates that Tre6P is not simply a signal of sucrose status, but also regulates the level of sucrose in the plant. Thus, Tre6P may be seen as part of a homeostatic mechanism to control the level of sucrose in plant cells, ensuring that it does not rise too high or drop too low, analogous to the control of blood glucose levels in animals by

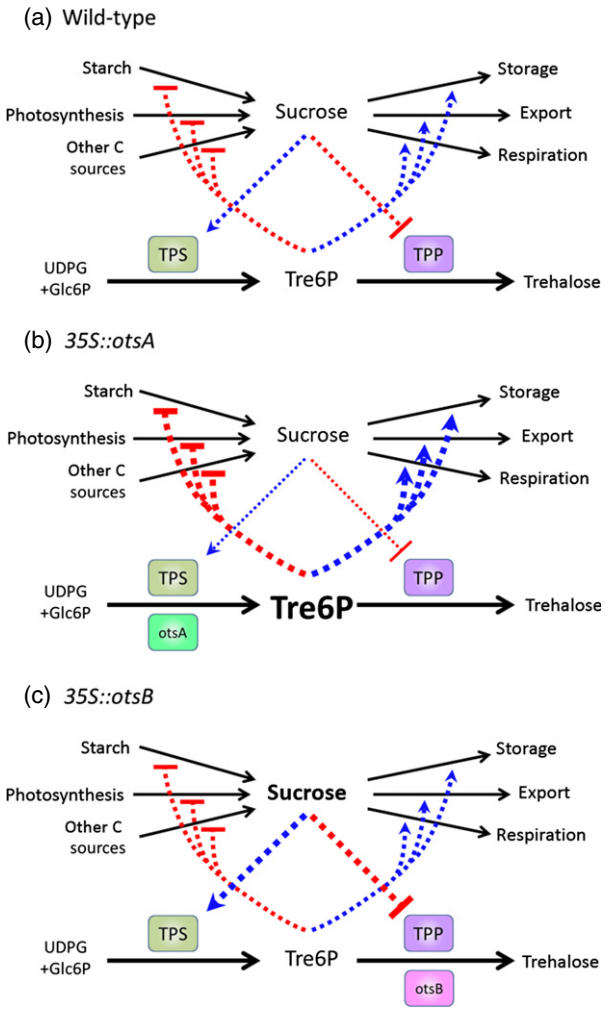

Figure 2. The Tre6P–sucrose nexus in wild-type, *35S::otsA* and *35S::otsB* plants.
Blue and red dashed lines show putative activation and inhibition, respectively. Some of the proposed effects of Tre6P on sucrose production and consumption are supported by experimental evidence, e.g. inhibition of the remobilization of starch to sucrose in leaves at night. Others are theoretical possibilities that have not yet been investigated. The relative effect of Tre6P on these processes varies between tissues and developmental stages, and depends on environmental conditions (e.g. light versus dark). In *35::otsA* plants, unregulated TPS (otsA) activity increases Tre6P and decreases sucrose levels, resulting in a high Tre6P:sucrose ratio. In *35S::otsB* plants, increased sucrose compensates for the constant depletion of Tre6P by the unregulated TPP (otsB), resulting in almost wild-type levels of Tre6P but a low Tre6P:sucrose ratio. For simplicity, the figure does not show other sucrose signalling pathways, such as translational regulation of the BZIP11 transcription factor by sucrose (Rahmani *et al.*, 2009; Ma *et al.*, 2011). Interactions between Tre6P-mediated sucrose signalling and other sucrose signalling pathways may be unbalanced by genetic perturbation of Tre6P levels.

glucagon and insulin. To elaborate, an increase in Tre6P is an indication of increasing sucrose levels, potentially triggering mechanisms to lower sucrose levels back to the optimal concentration range for the cell, by restricting sucrose synthesis and/or promoting sucrose consumption via catabolism, storage or export (Figure 2a). As sucrose returns to

the desired concentration, Tre6P also decreases. Parallel changes in sucrose and Tre6P levels occur in both source leaves (Lunn *et al.*, 2006; Nunes *et al.*, 2013c; Pal *et al.*, 2013; Sulpice *et al.*, 2014) and sink organs such as the *A. thaliana* shoot apex (Wahl *et al.*, 2013). Thus, Tre6P may be part of regulatory networks that regulate the provision of sucrose for export in source organs, and the use of sucrose in growing sink organs.

In *35S::otsA* plants (Figure 2b), high Tre6P triggers responses to lower the concentration of sucrose, but Tre6P levels do not fall back in parallel with sucrose due to the additional unregulated activity of the constitutively expressed heterologous TPS. The endogenous mechanisms that tightly link Tre6P and sucrose levels still operate, hence the strong correlation between these metabolites in the *35S::otsA* plants, but the plant is pushed into a new metabolic state with high Tre6P but lower than normal levels of sucrose, which are likely to be sub-optimal for growth.

Constant unregulated removal of Tre6P by the heterologous TPP in the *35S::otsB* plants may have been expected to reduce Tre6P below the levels seen in wild-type plants, but this was not the case. It appears that these plants adapt to the high and unregulated activity of the heterologous TPP by allowing sucrose levels to increase. Higher sucrose acts via the endogenous network to increase Tre6P levels, counteracting the downward pressure on Tre6P from unregulated TPP activity. Whether by chance or design, these opposing forces reach a balance in which the plants have very similar levels of Tre6P to wild-type plants, but elevated sucrose and a lower Tre6P:sucrose ratio (Figure 2c).

We are only beginning to understand the molecular mechanisms that maintain the nexus between Tre6P and sucrose. In *A. thaliana* seedlings, the response of Tre6P to exogenously supplied sucrose is inhibited by cycloheximide but insensitive to cordycepin, showing a dependence on *de novo* protein synthesis but not *de novo* transcription (Yadav *et al.*, 2014). Experiments with protein kinase and protein phosphatase inhibitors suggested that protein phosphorylation is also involved in the response (Yadav *et al.*, 2014). Destabilization of *AtTPS8–AtTPS11* transcripts and a small decrease in the polysome loading of *AtTPPJ* transcripts were observed after sucrose feeding, but it is not known whether these are significant factors in the sucrose-induced rise in Tre6P. These results implicate protein synthesis and phosphorylation in the sucrose-driven changes in Tre6P, but we do not yet know which proteins are involved.

In the sections below, we consider potential mechanisms for regulation of intracellular sucrose concentrations by Tre6P, via effects on photoassimilate partitioning between sucrose and starch during the day, conversion of starch to sucrose at night, and consumption of sucrose by growth.

REGULATION OF STARCH SYNTHESIS BY TRE6P

Plants acquire carbon via photosynthesis, which only occurs in the light. During the day, part of the photosynthate is used for synthesis and export of sucrose to support growth of sink organs. Most of the remainder is accumulated as starch in the chloroplast. Starch is remobilized at night and used to support respiratory metabolism in the leaf, or to synthesize sucrose that is exported to support growth of sink organs during the night. These transitory starch reserves enable the plant to cope with the daily challenge of surviving through the night, and must be prudently managed to optimize growth rates and achieve reproductive success (Smith and Stitt, 2007; Andriotis *et al.*, 2012; Stitt and Zeeman, 2012).

Photoassimilate partitioning is regulated by a complex network of regulatory mechanisms (MacRae *et al.*, 2006; Stitt *et al.*, 2010). As sucrose accumulates in leaves during the day, there is often a shift in partitioning into starch (Stitt *et al.*, 1987), although this was not observed in maize and wheat, which store sucrose in preference to starch in their leaves (Lunn and Hatch, 1997; Trevanion *et al.*, 2004). Growth of *A. thaliana* under short-day conditions (Sulpice *et al.*, 2014) or a sudden extension of the night (Gibon *et al.*, 2004) led to transient accumulation of sugars in the leaf at the start of the light period, because these treatments decrease growth in the dark and this is not immediately reversed upon illumination. Under both circumstances, the transient accumulation of sugars leads to stimulation of starch synthesis. At least two mechanisms may account for this stimulation of starch synthesis when sugars accumulate in the leaf: (i) feedback inhibition of sucrose synthesis leading to allosteric activation of ADP-glucose pyrophosphorylase (AGPase), and (ii) redox activation of AGPase.

In spinach (*Spinacia oleracea*), sucrose phosphate synthase becomes less activated as sucrose accumulates during the day, triggering feedback mechanisms such as accumulation of fructose-2,6-bisphosphate that limit the export of triose phosphates from the chloroplasts (Stitt *et al.*, 1988, 2010). This leads to an increase in the 3-phosphoglycerate to orthophosphate ratio in the chloroplast stroma, allosteric activation of AGPase, and stimulation of starch synthesis (Stitt *et al.*, 1987; Ballicora *et al.*, 2004). AGPase may also be activated by reversible reduction of a disulfide bridge between the two small subunits of the heterotetrameric holoenzyme (Ballicora *et al.*, 1999; Hädrich *et al.*, 2011). This activation is promoted by light or accumulation of sugars (Tiessen *et al.*, 2002; Hendriks *et al.*, 2003).

Kolbe *et al.* (2005) observed that incubation of isolated pea chloroplasts with dithiothreitol and 0.1–1.0 mM Tre6P in the dark led to substantial reduction (i.e. activation) of AGPase, whereas dithiothreitol plus sucrose or trehalose

had no effect. This led to the proposal that stimulation of starch synthesis by sugars is mediated by Tre6P via redox modulation of AGPase (Kolbe *et al.*, 2005). It was also noted that *35S::otsA A. thaliana* plants had a higher ratio of reduced AGPase to oxidized AGPase, and more starch than in wild-type plants (Schluepmann *et al.*, 2003; Kolbe *et al.*, 2005; Wingler *et al.*, 2012). The redox status of AGPase in *A. thaliana* rosettes changes in parallel with diurnal fluctuations in sucrose content and Tre6P, providing support to this hypothesis (Lunn *et al.*, 2006).

However, recent analysis of *A. thaliana* plants carrying an ethanol-inducible TPS (otsA) construct did not support a direct causal link between Tre6P and changes in the redox status of AGPase (Martins *et al.*, 2013). Ethanol-induced over-expression of TPS during the day increased Tre6P levels by up to 11-fold. This led to a small but transient increase in the rate of starch accumulation in the middle of the day, but no significant change in the redox status of AGPase compared with non-induced control plants (Martins *et al.*, 2013). Thus the correlation between Tre6P and the redox status of AGPase observed by Lunn *et al.* (2006) may be driven by parallel but independent responses to sucrose.

Hädrich *et al.* (2011) used site-directed mutagenesis to remove the cysteine residues (Cys81) involved in formation of the disulfide bridge in AGPase. Preventing redox modulation of the enzyme in this way had surprisingly little effect on the rate of starch synthesis, except under short-day conditions at low irradiance. A similar independent study also came to the conclusion that redox regulation of AGPase plays little role in setting the rate of starch synthesis (Li *et al.*, 2012). The contamination of pre-2011 Tre6P supplies from Sigma-Aldrich with the surfactant 2-hydroxyethyl 12-hydroxyoctadecanoate and various fatty acid esters (Yadav *et al.*, 2014) suggests that there may be an alternative interpretation for the reduction of AGPase observed in isolated chloroplasts when incubated with dithiothreitol and Tre6P (Kolbe *et al.*, 2005). The detergent-like nature of these contaminants may have increased the permeability of the chloroplast envelope, allowing entry of dithiothreitol from the external medium and thus reduction of AGPase. As the experiments lacked controls comprising Tre6P that had been pre-incubated with TPP (Kolbe *et al.*, 2005) to test the effect of any contaminants, it is not possible to eliminate this alternative explanation for the observed changes in the redox status of AGPase. It is also worth noting that the estimated cytosolic concentration of Tre6P in the light (4–7 μM; Martins *et al.*, 2013) is considerably below the range of concentrations (0.1–1.0 mM) at which Kolbe *et al.* (2005) observed effects of exogenous Tre6P on AGPase in their isolated chloroplast experiments.

The lack of an essential control in the isolated chloroplast experiments (Kolbe *et al.*, 2005), and the finding that loss of redox modulation of AGPase has little or no effect on starch synthesis (Hädrich *et al.*, 2011; Li *et al.*, 2012), weaken the evidence originally put forward in support of the hypothesis that Tre6P mediates stimulation of starch synthesis by sugars via redox modulation of AGPase. The hypothesis was directly tested *in vivo* by experiments with inducible TPS lines, which showed that a large induced increase in Tre6P resulted in only a small and transient stimulation of starch synthesis *in vivo*, which was independent of reductive activation of AGPase (Martins *et al.*, 2013). In conclusion, even if the effect of Tre6P on AGPase *in vitro* were confirmed using uncontaminated supplies of Tre6P and necessary controls (e.g. pre-incubation with TPP), there is little evidence that such a mechanism plays a substantial role in control of starch synthesis and photoassimilate partitioning *in vivo* under conditions that have been investigated to date.

REGULATION OF STARCH DEGRADATION BY TRE6P

In contrast to starch synthesis, the regulation of starch degradation is poorly understood. The circadian clock is known to play a critical role, enabling the plant to match the rate of starch degradation to the anticipated length of the night, but the precise mechanisms are not yet known (Weise *et al.*, 2006; Stitt *et al.*, 2007; Smith and Stitt, 2007; Graf *et al.*, 2010, Stitt and Zeeman, 2012; Graf and Smith, 2011; Scialdone *et al.*, 2013).

In experiments with inducible TPS lines, Martins *et al.* (2013) observed that a two- to threefold increase in Tre6P during the night led almost immediately to a significant inhibition of starch degradation and a two- to threefold decrease in sucrose. Given the strong dependence of Tre6P on sucrose, this suggested that, in wild-type plants, Tre6P may be part of a feedback mechanism to regulate the rate of starch degradation according to the demand for sucrose (Martins *et al.*, 2013). It was also noted that inhibition of starch degradation by high Tre6P may contribute to the high-starch phenotype of *A. thaliana* plants that constitutively over-express TPS (Schluepmann *et al.*, 2003; Kolbe *et al.*, 2005), and may be more important than any effects of Tre6P on starch synthesis (Martins *et al.*, 2013).

The mechanism by which Tre6P inhibits starch degradation is not yet known. Maltose is the main product of starch breakdown in *A. thaliana* leaves (Weise *et al.*, 2004; Niittylä *et al.*, 2004). Maltose levels rose fourfold within 2 h of dusk in non-induced control plants but remained low throughout the night in the induced plants with elevated Tre6P (Martins *et al.*, 2013), suggesting that an early step in the pathway of starch degradation in the chloroplasts was inhibited. Starch granules isolated from induced plants had a higher P_i content than granules from control plants, consistent with disruption of either the phosphorylation/dephosphorylation cycle required for starch degradation or inhibition of β-amylase, the main starch-hydrolysing enzyme in leaves. However, Tre6P did

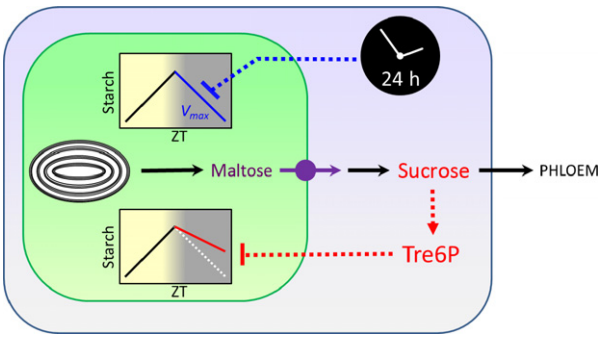

Figure 3. Control of starch breakdown by Tre6P and the circadian clock.
The maximum permissible rate of starch degradation is set by the circadian clock to ensure that starch reserves are not exhausted before the expected dawn. If sucrose export is restricted by low demand from sink organs, sucrose accumulates in the leaves and Tre6P increases, leading to inhibition of starch degradation. It is not known how the clock-derived signal is transmitted from the nucleus, where core components of the clock operate, to the chloroplasts. Tre6P probably inhibits starch breakdown via an intermediary that is formed in the cytosol and transmitted to the chloroplast.

not inhibit the *in vitro* activities of key starch-degrading enzymes *in vitro*, and an increase in the *in vivo* Tre6P level had no effect on the maximal catalytic activities or protein abundances of these enzymes. Given that Tre6P is predominantly, if not exclusively, located in the cytosol (Martins *et al.*, 2013), it is possible, even likely, that Tre6P does not inhibit starch degradation directly in the chloroplasts, but acts via a pathway that starts in the cytosol.

Martins *et al.* (2013) proposed that regulation of starch degradation by Tre6P is superimposed on regulation by the circadian clock (Figure 3). It is envisaged that the clock sets the maximum permissible rate of starch degradation to prevent the plant running out of starch before dawn, whilst sucrose-dependent changes in Tre6P modulate the rate of starch breakdown according to the demand for sucrose. If demand exceeds supply, sucrose levels decrease, triggering a decrease in Tre6P and allowing the rate of starch degradation to accelerate up to the maximum rate set by the clock. Conversely, if supply exceeds demand, Tre6P increases along with sucrose, reducing the rate of starch degradation. Co-regulation of starch degradation by Tre6P and the clock in this way enables the plant to make maximal use of its starch reserves for growth while avoiding the danger of running out of carbon before the end of the night.

CONTROL OF PLANT GROWTH AND DEVELOPMENT BY TRE6P

The role of trehalose metabolism in control of plant growth and development has been reviewed extensively (Ramon and Rolland, 2007; Paul *et al.*, 2008, 2010; Smeekens *et al.*, 2010; Ponnu *et al.*, 2011; Nunes *et al.*, 2013c; O'Hara *et al.*, 2013; Lastdrager *et al.*, 2014). We give a brief overview of the wide range of metabolic, growth and developmental traits that are altered in transgenic lines with modified levels of Tre6P (Table 1), and then discuss hypotheses about how Tre6P may cause these changes.

Arabidopsis thaliana tps1-1 and *tps1-2* null mutants are severely impaired in their ability to complete embryogenesis (Eastmond *et al.*, 2002; Gómez *et al.*, 2006). Their embryos accumulate sugars and starch but have less protein than wild-type embryos, show abnormal cell-wall formation, and undergo fewer cell divisions (Gómez *et al.*, 2006). However, patterning and cell differentiation are similar to wild-type embryos at the same developmental stage, and programs for lipid storage and seed/embryo desiccation are completed. The *tps1* mutants were complemented by expression of AtTPS1, and to a large extent by expression of a heterologous TPS from *E. coli* (otsA; Eastmond *et al.*, 2002). This suggests that loss of Tre6P synthetic capacity is the main cause of embryo arrest in the *tps1* mutants, but non-catalytic functions for the AtTPS1 protein cannot be entirely excluded (Geelen *et al.*, 2007).

During vegetative growth, *A. thaliana 35S::otsA* lines have higher Tre6P and starch content than wild-type plants, but lower sucrose, glucose 1-phosphate (Glc1P) and glucose 6-phosphate (Glc6P; Yadav *et al.*, 2014). Anthocyanin content is increased in *35S::otsA* plants. They have smaller leaves (Schluepmann *et al.*, 2003), at least partly due to changes in leaf composition, including a lower specific leaf area (i.e. less area per unit fresh weight) and dry weight content per unit fresh weight (Yadav *et al.*, 2014). There are broadly opposite responses in *A. thaliana 35S::otsB* plants, except that Tre6P content is indistinguishable from that of wild-type plants, or is only marginally decreased with respect to wild-type plants (see above). After embryo rescue by dexamethasone-inducible or embryo-specific expression of *AtTPS1*, null *tps1* mutants grow very slowly and are almost infertile (van Dijken *et al.*, 2004; Gómez *et al.*, 2010). In growing potato tubers, increased or decreased Tre6P levels lead to lower tuber size and yield. Tubers with elevated Tre6P have lower levels of sucrose and hexose phosphates, decreased starch, higher respiration and more lenticels (Debast *et al.*, 2011).

There is growing evidence that Tre6P regulates developmental programs. In *A. thaliana*, increased Tre6P leads to early flowering, while decreased Tre6P delays flowering (Schluepmann *et al.*, 2003). Tre6P modulates the canonical *CONSTANS (CO)/FLOWERING LOCUS T (FT)* photoperiod flowering pathway and the miR156/*SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL3)* maturity pathway (Wahl *et al.*, 2013). Loss of the RAMOSA3 isoform of TPP in maize inflorescence primordia leads to abnormal branching of the inflorescences (Satoh-Nagasawa *et al.*, 2006). After floral induction, *A. thaliana 35S::otsA* lines exhibit increased inflorescence branching and high rates of seed abortion (Schluepmann *et al.*, 2003), while senescence is delayed in *35S::otsB* lines (Wingler *et al.*, 2012).

In potato tubers, increased Tre6P strongly delays sprouting, which may be related to decreased catabolism of abscisic acid (ABA; Debast *et al.*, 2011).

There is emerging evidence for cell-specific actions of Tre6P. One example is during flowering, where Tre6P interacts with the companion cell-specific *CO*/*FT* pathways, and *TPS1* transcripts are detectable only in specific zones of the shoot apex (Wahl *et al.*, 2013). Another example is *A. thaliana* guard cells, in which there is specific or particularly high expression of *AtTPPG* and *AtTRE1*, implicating Tre6P/trehalose metabolism in control of stomatal conductance (Vandesteene *et al.*, 2012; van Houtte *et al.*, 2013).

Some of these phenotypic responses fit readily into the concept that Tre6P is a sucrose signal. One example is the inhibition of starch breakdown by Tre6P (see above). Another is the regulation of flowering, in which Tre6P may be viewed as an input into environmental (*CO*/*FT*) and developmental (miR156/*SPL3*) signalling pathways such that flowering is delayed when less carbon is available (Wahl *et al.*, 2013). Thus, the *CO*/*FT* pathway triggers flowering as the day length increases, and this response is attenuated when sucrose is low because Tre6P (and/or AtTPS1) is required for induction of *FT* by *CO* (Wahl *et al.*, 2013). Under short-day conditions, flowering is induced by the maturity pathway, with a gradual decrease of miR156 levels leading to induction of flowering by de-repression of *SPL3*. Under such conditions, sugars (Yang *et al.*, 2013; Yu *et al.*, 2013), possibly acting via Tre6P (Wahl *et al.*, 2013), accelerate the decrease in miR156, leading to increased expression of *SPL3* and earlier induction of flowering. Such responses link floral induction to the plant's internal resources, accelerating flowering when carbon is available and delaying it when carbon is in short supply.

However, other phenotypic responses (Table 1) fit less readily into this simple concept. One example is the apparent stimulation of photosynthesis in *35S::otsA* lines, which appears to contradict the well-established repression of photosynthesis by high-sugar signals (Paul and Foyer, 2001; Smeekens *et al.*, 2010). Other examples are the slower growth of *35S::otsA* lines (Yadav *et al.*, 2014), and the contrast between earlier flowering and increased branching but decreased seed set in *35S::otsA* lines (Schluepmann *et al.*, 2003). These diverse, and sometimes contradictory, phenotypes may arise because metabolic, physiological and developmental processes are unbalanced by genetic interventions in Tre6P metabolism. This may occur in at least three ways.

The first relates to perturbation of the link between the levels of Tre6P and sucrose as discussed above. A consistent result across all studies on mutants and transgenic plants with altered expression of *TPS* and *TPP* is that an increase in Tre6P is accompanied by a decrease in sucrose, while a decrease, or attempted decrease, in Tre6P leads to an increase in sucrose levels (Figure S1). The changes in sucrose are accompanied by changes in the levels of hexose phosphates (Table 1), which are the precursors for sucrose synthesis and the products of sucrose degradation. As already discussed, Tre6P is probably a key participant in a network that regulates the synthesis and use of sucrose, and hence the sucrose concentration within the cell. Genetic interventions that increase Tre6P synthesis or Tre6P degradation throw this network off balance, creating a very confusing situation, both for the plant and the researcher. For example, over-expression of TPS increases Tre6P, which signals to the plant that sucrose is high, but, in reality, the levels of sucrose (and hexose phosphates) are decreased and probably trigger contrary signals. Such imbalances may transiently occur in wild-type plants when environmental conditions are changing, but may be corrected by re-adjusting Tre6P levels to match sucrose levels. In line over-expressing TPS and TPP, this adjustment is blocked and the plant becomes chronically confused.

A second reason is the multi-layered nature of the affected traits, which may lead to apparently conflicting results. One example is the apparent increase in photosynthesis in *35S::otsA* lines (Table 1). This may be partly due to changes in leaf morphology and composition, including the greater leaf thickness, which tends to increase the maximum rate of photosynthesis on a leaf area basis (Yadav *et al.*, 2014). The opposite changes in Tre6P and sucrose levels in *35S::otsA* plants may also be a contributory factor, especially if photosynthesis responds to other sucrose-dependent signals in addition to Tre6P.

A third reason for the conflicting phenotypes may be that Tre6P regulates developmental transitions that lead to an irreversible change in the demand for carbon, and that inappropriate regulation due to genetic clamping of Tre6P levels results in imbalances between the supply and demand for sucrose at a later point in the life cycle of the plant. One such example is flowering; over-expression of TPS leads to an over-optimistic assessment of the available carbon resources by the plant, leading to precocious flowering and a consequent demand for carbon that cannot be met by the resources that are actually available.

When investigating the mode of action of Tre6P, it is important to design experiments to minimize the impacts of these secondary and potentially confounding responses. Thus, whilst *35S::otsA* lines grow more slowly (Yadav *et al.*, 2014), and *35S::otsB* lines are larger than wild-type *A. thaliana* (Schluepmann *et al.*, 2003), interpretation of these apparent changes in growth must take into account confounding effects of changes in flowering time (Wahl *et al.*, 2013) and leaf structure (Yadav *et al.*, 2014). Quantitative growth analysis actually showed that *35S::otsB* lines do not grow faster than wild-type plants (Yadav *et al.*, 2014). Over-expression of *otsA* and *otsB* in potato tubers led to smaller tubers in both cases, but probably for different reasons; high Tre6P appears to lead to higher

rates of respiration resembling those seen after heterologous over-expression of invertase and hexokinase (Trethewey *et al.*, 1998, 1999), while lower Tre6P appears to interfere with the tuber's ability to use sucrose. Interpretation of correlative studies in which environmental or physiological perturbations are used to perturb Tre6P levels (Nunes *et al.*, 2013a; Sulpice *et al.*, 2014) is difficult because many other metabolites correlate with Tre6P and hence with growth. This leaves open the question of whether the observed correlations between Tre6P and growth are causal or coincidental. To disentangle these complex interactions, it will be necessary to use more targeted expression studies, including the use of induced TPS/TPP expression with dense sampling times. Even then, it may still prove difficult to disentangle primary and secondary responses to a change in Tre6P, as demonstrated by induced increases in Tre6P at night that led to an almost immediate inhibition of starch degradation and a decrease in sucrose levels (Martins *et al.*, 2013).

INTERACTION BETWEEN TRE6P AND SNRK1

One proposed, and widely discussed, mode of action for Tre6P involves inhibition of SnRK1 (Zhang *et al.*, 2009; Smeekens *et al.*, 2010; Nunes *et al.*, 2013b; O'Hara *et al.*, 2013). SnRK1 belongs to a widespread family of eukaryotic protein kinases (e.g. Sucrose Non-Fermenting 1 in yeast and AMP-dependent kinase in mammals) that are involved in energy sensing and homeostasis, and is also involved in stress responses in plants (Baena-Gonzalez *et al.*, 2007).

In vitro experiments in crude extracts from developing *A. thaliana* tissues showed that Tre6P inhibits SnRK1 (Zhang *et al.*, 2009). Such inhibition was also observed with SnRK1 that had been partially purified by immunoprecipitation but was dependent on re-addition of the SnRK1-depleted supernatant from the immunoprecipitation reaction, indicating that some additional factor, so far unidentified, is required to mediate the inhibition by Tre6P (Zhang *et al.* 2009; Nunes *et al.*, 2013a,b,c). A similar inhibition of SnRK1 was found in potato tubers (Debast *et al.*, 2011). A possible caveat is that inhibition of SnRK1 *in vitro* has not yet been confirmed using a demonstrably uncontaminated source of Tre6P. *A. thaliana* SnRK1 is also inhibited by Glc1P and Glc6P (Nunes *et al.*, 2013b).

The K_i value for inhibition of SnRK1 by Tre6P (5 μM; Nunes *et al.*, 2013b) lies within the range of *in vivo* Tre6P concentrations (4–7 μM) estimated in *A. thaliana* rosettes in the light (Martins *et al.*, 2013). The total Tre6P content of sucrose-fed seedlings was similar to that in illuminated rosettes, suggesting that *in vivo* concentrations are in a comparable range in this material (Lunn *et al.*, 2006). However, Tre6P levels were threefold lower in rosettes harvested in the dark, and more than 10 fold lower in carbon-starved rosettes and seedlings (Lunn *et al.*, 2006; Carillo *et al.*, 2013; Yadav *et al.*, 2014). The K_i values for Glc1P and Glc6P (55 and 300 μM; Nunes *et al.*, 2013b) lie at the lower end of the concentration range estimated for these metabolites in darkened or carbon-starved plants (20–300 and 200–2500 μM respectively; Arrivault *et al.*, 2009; Martins *et al.*, 2013; Yadav *et al.*, 2014). While such comparisons involve assumptions, SnRK1 may be inhibited by Glc1P and Glc6P except under conditions of carbon starvation, but is only inhibited by Tre6P under carbon-replete conditions. As noted by Nunes *et al.* (2013b), there may be a synergistic interaction between Glc1P and Tre6P. However, it is not clear whether this modifies the K_i for Tre6P.

Figure 4 presents two scenarios for how the interaction between Tre6P and SnRK1 may operate *in vivo*. In one scenario, which has been extensively discussed (Zhang *et al.*, 2009; Smeekens *et al.*, 2010; Nunes *et al.*, 2013c; O'Hara *et al.*, 2013), Tre6P acts primarily by inhibiting SnRK1. This provides an attractively simple framework to understand how an increase in the sucrose supply may inhibit catabolism and activate biosynthesis and growth processes. In the other scenario, Tre6P and SnRK1 act in separate pathways with distinct, but possibly overlapping, targets, and an interaction between the two pathways is mediated via the inhibitory action of Tre6P on SnRK1.

The experimental evidence that Tre6P acts primarily by inhibiting SnRK1 *in vivo* is based on patterns of differential gene expression in wild-type *A. thaliana* plants versus *35S::otsA* lines (Zhang *et al.*, 2009; Paul *et al.*, 2010; Wingler *et al.*, 2012), which showed a qualitative overlap (50% of differentially expressed genes) with the changes in gene expression after transient over-expression of SnRK1 in *A. thaliana* mesophyll protoplasts (Baena-Gonzalez *et al.*, 2007). The differentially expressed genes in wild-type versus *B33::otsB* potato tubers, which had increased

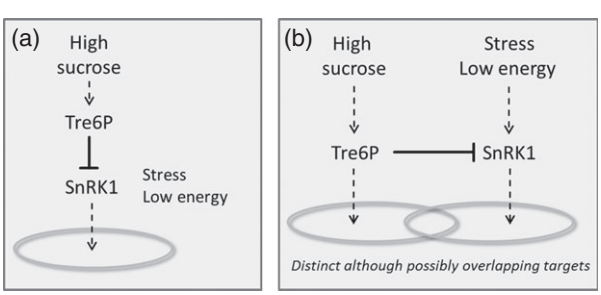

Figure 4. Scenarios for the interaction between Tre6P and SnRK1 *in vivo*.
(a) Tre6P acts primarily via inhibition of SnRK1. In this scenario, SnRK1 is a key component in a stress and low-energy signalling pathway that activates catabolism and represses processes required for growth. Tre6P provides a sucrose-dependent input to the SnRK1 pathway, attenuating it when sucrose is high and enhancing it when sucrose is low.
(b) SnRK1 and Tre6P act in separate pathways that interact via Tre6P-mediated inhibition of SnRK1. SnRK1 is a key component in a stress and low-energy signalling pathway that activates catabolism and represses growth. Tre6P is a signal metabolite in a pathway that mediates sucrose-dependent regulation of metabolism, development and growth. Inhibition of SnRK1 by Tre6P provides sucrose-dependent input into the SnRK1 pathway.
These two scenarios are extremes; intermediate scenarios may be envisaged.

TPP activity specifically in the tubers, showed a 20% overlap with those in SnRK1-expressing *A. thaliana* protoplasts (Debast *et al.*, 2011). In an analogous approach, Nunes *et al.* (2013a) reported that the transcript abundance for a subset of SnRK1 reporter genes correlated with Tre6P levels in a range of treatments that modify Tre6P levels, including sugar feeding, low nitrogen and low temperature.

Although superficially compelling, we suggest that these transcriptomic comparisons are inconclusive for the following reasons. First, there is incomplete overlap (only 20–50% qualitative agreement) between the transcript responses to altered Tre6P levels and SnRK1 over-expression. This discrepancy may be because the experiments on Tre6P and SnRK1 responses were performed in different biological systems. However, it underlines the need for experiments that define the transcriptomic response to SnRK1 in a similar experimental system to that being used to study the response to Tre6P. Second, in all comparisons that use expression data from *35S::otsA* or *B33::otsB* lines, it is impossible to know which of the changes are a direct response to Tre6P, and which are secondary or indirect responses to the long-term perturbation of Tre6P levels. This question may probably only be answered by using inducible systems to alter Tre6P levels in a transient manner. Third, firm conclusions about the mode of action of Tre6P cannot be drawn from correlative studies (e.g. Wingler *et al.*, 2012; Nunes *et al.*, 2013a) because it is impossible to determine whether the observed changes in gene expression are due to alterations in the levels of Tre6P or to changes in the levels of sucrose, or other related metabolites (see above). The correlations between the level of Tre6P and the various SnRK1 reporter transcripts are also often driven by comparison across treatments rather than within a given treatment, where Tre6P levels are only poorly correlated with expression of SnRK1 reporter genes.

As a more general point, proof that Tre6P acts primarily by inhibiting SnRK1 requires investigation of the primary action of SnRK1, i.e. the phosphorylation status of SnRK1 target proteins, rather than relying on distal changes in gene expression. Changes in transcript abundance are a less direct readout of SnRK1 activity than protein phosphorylation, and are potentially unreliable if the gene's expression is also regulated by other factors, i.e. transcripts may not be specific reporters for SnRK1 activity. It will also be important to apply classical genetic approaches to show that mutants with altered levels of Tre6P and altered SnRK1 function have strictly non-additive phenotypes. Further, this simple hypothesis must explain why growth is negatively correlated with Tre6P in *35S::otsA* plants compared with *35S::otsB* and wild-type *A. thaliana* plants (Schluepmann *et al.*, 2003; Yadav *et al.*, 2014), or when wild-type *A. thaliana* plants are grown under various conditions (Schluepmann *et al.*, 2004; Nunes *et al.*, 2013a,b,c), and also in potato tubers expressing *otsA* or *otsB* compared to wild-type tubers (Debast *et al.*, 2011).

In light of the weak, and sometimes conflicting, experimental support for scenario one (Figure 4a), we propose an alternative scenario (Figure 4b), in which Tre6P acts in a separate pathway from SnRK1, but interacts with the SnRK1 pathway. This concept has not, to our knowledge, been experimentally investigated, but we argue that this scenario fits better with several features of trehalose metabolism and the diversity of phenotypes attributed to Tre6P signalling. First, the diversity of phenotypes, including effects on stomatal conductance, flowering time, maize inflorescence development and tuber sprouting, argues against a 'one size fits all' hypothesis for Tre6P signalling, and appears more likely to involve dedicated signalling pathways. Second, the expansion of class II *TPS* and *TPP* genes during plant evolution and their profoundly differing expression patterns (Lunn, 2007; Avonce *et al.*, 2010; Vandesteene *et al.*, 2012) are consistent with Tre6P signalling pathways being specifically tailored to different tissue types and stages of development. Third, this scenario provides a rich and more flexible network in which information about the availability of sucrose, the main transport sugar in plants, may be integrated with other forms of energy and resource signalling that are envisaged to be mediated by the SnRK1 pathway. Tre6P tracks sucrose across a very wide dynamic range, increasing in tandem even as sucrose levels rise far above those found during starvation (Lunn *et al.*, 2006; Martins *et al.*, 2013; Nunes *et al.*, 2013a; Yadav *et al.*, 2014). This makes it unlikely that Tre6P signalling is simply reporting carbon starvation.

The alternative scenario may be readily extended to include the recently established inhibitory action of Glc1P and Glc6P on SnRK1 (see above). In conditions under which sucrose, Tre6P and hexose phosphate levels change in the same direction, Tre6P, Glc6P and Glc1P act in unison to increase or decrease SnRK1 activity, thereby affecting the consumption of sucrose by restricting or promoting growth. However, hexose phosphate and sucrose levels sometimes change reciprocally in leaves (Stitt, 1991; Stitt *et al.*, 1983; Pal *et al.*, 2013; Martins *et al.*, 2013) and potato tubers (Geigenberger and Stitt, 1993; Tiessen *et al.*, 2002). In such situations, Tre6P and hexose phosphates act antagonistically on SnRK1 activity, providing a potential mechanism to rebalance sucrose metabolism and glycolysis. This interaction is disturbed in TPS and TPP over-expressing lines, providing a possible explanation for the displacement of sucrose and hexose phosphate levels and disturbance of metabolic balances in these plants.

To distinguish between the two scenarios in Figure 4, it is important to identify the immediate consequences of a change in Tre6P levels. If Tre6P acts only, or predominantly, via inhibition of SnRK1, the immediate downstream

responses to Tre6P and SnRK1 signalling will be similar. Nevertheless, subtle differences may be expected unless Tre6P is the only or dominant regulator of SnRK1, which appears unlikely given the wider scope of SnRK1 in signalling of energy and nutrient status in other life forms. Definition of distinct phenotypes should be possible if Tre6P and SnRK1 signalling involves separate pathways that are linked via the inhibitory action of Tre6P on SnRK1. Identification of specific phenotypes will make it possible to establish forward genetic screens using phenotypes that are unambiguously linked to Tre6P signalling. Based on current knowledge (Table 1), these may include starch degradation and flowering, but also more subtle phenotypes. Recent advances in next-generation sequencing to detect polymorphisms and link them to phenotypes (Schneeberger and Weigel, 2011; Nordström *et al.*, 2013) have greatly increased the speed of gene mapping, and allow mapping to be performed in the same genetic background as the mutant, which makes it easier to score subtle phenotypes.

TREHALOSE METABOLISM AND ABIOTIC STRESS TOLERANCE

In the sections below, we describe how plant trehalose metabolism is affected by various types of stress, discuss its role in the plant's adaptation to unfavourable growth conditions, and consider the prospects for engineering endogenous plant trehalose metabolism to improve stress tolerance in crop plants.

Temperature stress

Exposure to temperature extremes triggers complex physiological and biochemical responses in plants, including up- or down-regulation of many transcripts and proteins, changes in metabolite content including sugars, lipids and secondary metabolites, and modification of membrane composition and structure (Sanghera *et al.*, 2011). Transcriptomic and metabolomic analyses of cold- or heat-stressed plants have revealed changes in expression of *TPS* and *TPP* genes, and pinpointed trehalose as a putative compatible solute that may act in combination with other solutes during induction of thermotolerance (Kaplan *et al.*, 2004; Usadel *et al.*, 2008). However, it should be noted that low and high temperature may lead to changes in sucrose levels, and it is therefore important to distinguish between direct effects of temperature and secondary responses triggered by changes in sucrose levels.

Genetic evidence for a role of trehalose metabolism at high temperature was provided by the finding that AtTPS5 interacts with MULTIPROTEIN BRIDGING FACTOR 1c (MBF1c), which is a key regulator of thermotolerance, and that tolerance to high temperature is impaired in *A. thaliana tps5* null mutants (Suzuki *et al.*, 2008). There is also correlative evidence for a role of trehalose metabolism at low temperature. In rice, both *OsTPP1* and *OsTPP2* levels increased during cold stress treatment (Pramanik and Imai, 2005; Shima *et al.*, 2007). Similar results were obtained upon chilling stress in grapevine, with *VvTPPA* being induced to varying degrees in different organs (Fernandez *et al.*, 2012). In *A. thaliana*, *AtTPPA* was induced upon cold stress, resulting in increased trehalose and Tre6P levels (Iordachescu and Imai, 2008). However, there was a clear correlation between the Tre6P levels and sucrose content in all plant organs, which suggests that cold-induced accumulation of sucrose may underlie the rise in Tre6P levels (Fernandez *et al.*, 2012; Nunes *et al.*, 2013a).

Oxidative stress

Abiotic and biotic stress trigger accumulation of reactive oxygen species (ROS), which may have both positive and negative effects for the plant. ROS act as signalling molecules to regulate various processes such as pathogen defence and programmed cell death (Grant and Loake, 2000; Dangl and Jones, 2001). As ROS may accumulate to toxic levels, they must be scavenged to restrict oxidative damage. For this purpose, plants are equipped with various defence mechanisms, including accumulation of sugars.

There is *in vitro* and *in vivo* evidence that trehalose protects against hydroxyl radicals (Roitsch, 1999; Couee *et al.*, 2006). Over-expression of yeast *TPS1* in tobacco and tomato (*Solanum lycopersicum*) increased tolerance to oxidative stress induced by methyl viologen (Romero *et al.*, 2002; Cortina and Culianez-Macia, 2005). In addition, millimolar concentrations of trehalose have been shown to protect superoxide dismutase activity from heat inactivation *in vitro*. This mechanism may also contribute to the protective effect of trehalose against free radicals generated by heat stress in wheat, in addition to direct scavenging by trehalose (Luo *et al.*, 2008).

Hypoxia

Flooding is a significant problem for many plants, leading to hypoxia or even anoxia. Tre6P levels decreased in wild-type *A. thaliana* under low oxygen conditions, but not in plants engineered to over-express a non-symbiotic haemoglobin (Thiel *et al.*, 2011). However, it is unclear whether the observed changes in Tre6P are simply a response to hypoxia-driven changes in sucrose levels, or whether these play a role in adjusting the plant's metabolism and growth to decrease oxygen consumption and so avoid becoming completely anoxic.

Salt stress

Soil salinity presents an increasing threat to agriculture, and trehalose influences many processes that provide an advantage for plant survival under salt stress (Garcia *et al.*, 1997). Low to moderate levels of exogenous trehalose reduce Na^+ accumulation, whereas higher levels prevent NaCl-induced loss of chlorophyll in leaves and preserve

root integrity (Garcia *et al.*, 1997). Trehalose also accumulates in a range of wheat cultivars under salt stress, potentially due to enhanced TPS activity (El-Bashiti *et al.*, 2005). In rice, *OsTPP1* was transiently induced during salt stress, similar to the response upon chilling stress (Pramanik and Imai, 2005; Shima *et al.*, 2007). In *Medicago truncatula*, trehalase expression is down-regulated under salt stress (López *et al.*, 2008a,b). This allows trehalose accumulation, consistent with a role for this disaccharide as a protective agent against salt stress. In contrast, in a closely related species, alfalfa (*Medicago sativa*), the role of trehalose in osmoregulation has been questioned, as its concentration does not increase substantially upon salt stress (Fougere *et al.*, 1991). It should be noted that, even though there were large relative increases in trehalose content, absolute levels were still very low and probably had little direct protective effect against salt stress.

Drought stress

Some desiccation-tolerant resurrection plants, e.g. *S. lepidophylla*, *Myrothamnus flabellifolius* and *Sporobolus* spp. accumulate massive amounts of trehalose in response to drought, and may persist in metabolic stasis for several years until re-watered (Iturriaga *et al.*, 2006). Resurrection plants often also contain high levels of sucrose. Together these disaccharides are thought to stabilize membranes, proteins and other cellular components during stasis (Drennan *et al.*, 1993).

Trehalose levels are much lower in crop plants. A slight increase in trehalose content was nevertheless seen in drought-tolerant wheat and cotton (*Gossypium hirsutum*) varieties grown under water stress, coinciding with higher TPS expression (El-Bashiti *et al.*, 2005; Kosmas *et al.*, 2006). In some varieties of wheat, a decrease in trehalase activity also contributed to the increase in trehalose (El-Bashiti *et al.*, 2005). However, it remains unclear whether these low levels and small changes in trehalose have much protective effect. In a drought-resistant transgenic potato line expressing the yeast gene *ScTPS1*, there were substantial increases in other compatible solutes, including proline, inositol and raffinose, compared to wild-type (Kondrák *et al.*, 2012).

OPTIMIZING PLANT STRESS TOLERANCE BY MODIFICATION OF ENDOGENOUS TREHALOSE BIOSYNTHESIS PATHWAYS

Co-expression of heterologous TPS and TPP, or introduction of an artificial gene construct encoding a fused TPS–TPP enzyme, gave rise to tobacco and rice plants that had improved stress tolerance but no obvious morphological defects (Garg *et al.*, 2002; Lee *et al.*, 2003; Miranda *et al.*, 2007). These results provide further evidence that de-regulation of Tre6P levels is responsible for the aberrant phenotypes observed in earlier studies (see above).

Several approaches have been adopted to improve drought tolerance without the deleterious effects arising from constitutive expression of microbial *TPS* and *TPP* genes (Table 1). Expression of yeast *ScTPS1* in potato under the control of a drought-inducible promoter improved drought tolerance without large phenotypic side-effects (Stiller *et al.*, 2008; Kondrák *et al.*, 2012). In other studies, expression of endogenous trehalose-metabolizing enzymes has been manipulated to improve drought tolerance. For instance, constitutive expression of *AtTPS1* in *A. thaliana* improved drought tolerance with no visible effect on the plant's morphology, except for some delay in flowering (Avonce *et al.*, 2004). In a comparable study in rice, over-expression of *OsTPS1* improved the tolerance to cold, salinity and drought, with no other phenotypic alterations (Li *et al.*, 2011).

There are several possible explanations for the phenotypic differences in plants engineered to over-express endogenous versus heterologous TPS enzymes. Unlike the microbial enzymes, the over-expressed plant TPS enzymes have inherently lower activity due to the presence of the N-terminal auto-inhibitory domain, and are presumably still subject to some control by endogenous regulatory mechanisms, potentially moderating the metabolic disturbance. Another possibility is that the plant TPS proteins have 'moonlighting' functions that are independent of their catalytic activity, for example as transcriptional regulators or scaffolds for formation of protein complexes (Geelen *et al.*, 2007).

Despite the increase in abiotic stress tolerance, over-expressing endogenous genes generally resulted in only a minor increase in trehalose levels (Avonce *et al.*, 2004; Li *et al.*, 2011). Interestingly, over-expression of the plant's own *TPS* genes resulted in induction of stress-associated genes, including genes involved in ABA and glucose signalling pathways (Avonce *et al.*, 2004; Ramon *et al.*, 2007). This suggests that even minor changes in the level of trehalose and/or Tre6P may trigger abiotic stress responses, potentially making the plants more tolerant when subsequently exposed to stress conditions.

As an alternative approach to increase trehalose levels, van Houtte *et al.* (2013) used a reverse genetics approach to suppress the endogenous trehalase, *AtTRE1*, in *A. thaliana*. The resulting *tre1-1* null mutant and *tre1-2* knockdown mutant both accumulated higher levels of trehalose, but surprisingly were less drought tolerant than wild-type plants (van Houtte *et al.*, 2013). Conversely, over-expression of *AtTRE1* in the *tre1-3*^{OE} mutant and two *35S*::*AtTRE1* lines reduced trehalose content but enhanced drought tolerance. These results show that the small increases in trehalose in plants over-expressing TPS and TPP were not responsible for the improvements in drought tolerance seen in those plants, and, except for resurrection species, cast further doubt on trehalose

being a quantitatively important compatible solute in plants.

TREHALOSE METABOLISM AND STOMATAL CONDUCTANCE

Several of the anomalous observations about responses to abiotic stress may be explained by emerging evidence that trehalose metabolism plays an important role in control of stomatal conductance and water-use efficiency (Figure 5). Stomata in leaves of the *A. thaliana tps1-12* mutant have a smaller aperture than those in wild-type plants (Gómez *et al.*, 2010). Using promoter–reporter gene constructs, it was shown that expression of *AtTPPG* and *AtTRE1* is prominent in the guard cells of *A. thaliana* leaves (Vandesteene *et al.*, 2010; van Houtte *et al.*, 2013). Furthermore, AtTPS1 protein may be present at relatively high levels in stomatal cells compared to many other cell types or tissues in *A. thaliana*. AtTPS1 has rarely been detected in proteomic surveys of *A. thaliana* cells (http://suba.plantenergy.uwa.edu.au/; Tanz *et al.*, 2013), with guard cells being one of the few exceptions (Zhao *et al.*, 2008).

During drought stress, plants attempt to close their stomata to minimize water loss from the leaves. This response is generally triggered by ABA. *A. thaliana tppg*, *tre1-1* and *tre1-2* mutants fail to close their stomata when treated with exogenous ABA, showing that *AtTPPG* and *AtTRE1* are essential for ABA-mediated stomatal closure (Vandesteene *et al.*, 2010; van Houtte *et al.*, 2013). Further, *AtTRE1*-over-expressing plants were hypersensitive to exogenous ABA. They also had lower stomatal conductance than wild-type plants under non-drought conditions, possibly due to increased responsiveness to endogenous ABA (van Houtte *et al.*, 2013). A mechanistic link between ABA and trehalose metabolism is provided by the finding that ABA induces *AtTRE1* expression (van Houtte *et al.*, 2013). While the mechanism of transcriptional regulation by ABA is not yet established, the *AtTRE1* promoter has possible binding sites for MYB4, a transcription factor that is known to be induced under environmental stress conditions (Chen *et al.*, 2002), and also contains a W-box motif that is implicated in binding of MYB102 or WRKY transcription factors (O'Connor *et al.*, 2005; van Houtte *et al.*, 2013).

These results establish that trehalose metabolism plays an essential role in the responsiveness of guard cells to ABA and the control of stomatal conductance. Although the underlying signalling mechanisms are not yet defined, there is evidence for multiple interactions, with expression of *AtTRE1* being transcriptionally regulated by ABA, while both AtTPPG and AtTRE1 are essential for ABA to induce stomatal closure. Stomatal sensitivity to perturbation of trehalose metabolism may resolve the paradox that *tre1* mutant plants with elevated trehalose are more drought-sensitive than wild-type plants, while *AtTRE1* over-expressing plants with less trehalose are more drought-tolerant than the wild-type. It is important to further investigate the role of AtTPPG and possible changes in trehalose levels in facilitating or modulating the ABA signalling pathway. Such studies may provide insights into the molecular basis of the interaction between ABA signalling and Tre6P or sugar signalling, which has also been observed in other contexts (Avonce *et al.*, 2004) including potato dormancy (Debast *et al.*, 2011) and seedling germination (Smeekens *et al.*, 2010; Vandesteene *et al.*, 2012).

TREHALOSE IN PLANT–MICROBE AND PLANT–INSECT INTERACTIONS

The responses of plants to abiotic and biotic stresses, such as pathogen attack and insect herbivory, have several

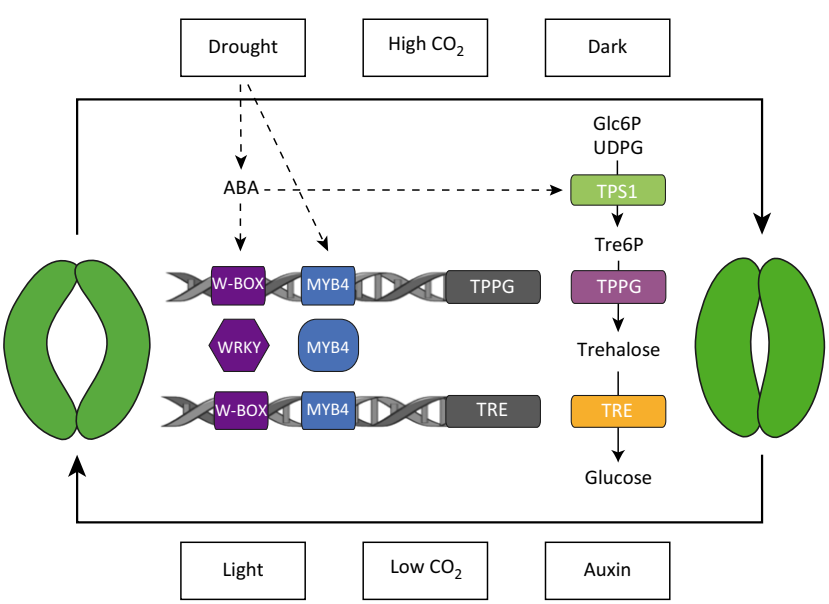

Figure 5. Trehalose metabolism and interaction with ABA signalling in guard cells.
A. thaliana trehalase1 (*tre1*) mutants accumulate more trehalose than wild-type plants, but are less drought-tolerant because the stomata are no longer sensitive to ABA (van Houtte *et al.*, 2013). ABA-triggered closure of stomata is also blocked in the *tppg* mutant. Expression of *AtTPS1*, *AtTPPG* and *AtTRE1* is regulated by drought and ABA, with that of *AtTPPG* and *AtTRE1* possibly being regulated via transcriptional regulation by WRKY and MYB transcription factors (Vandesteene *et al.*, 2012; van Houtte *et al.*, 2013). It is not yet known whether trehalose metabolism is necessary for stomatal responses to other environmental stimuli, such as light and CO_2 concentrations.

features in common: involvement of phytohormones, especially ABA, jasmonic acid and ethylene, generation of ROS, and changes in primary and secondary metabolism (Laloi *et al.*, 2004; Mauch-Mani and Mauch, 2005; Fujita *et al.*, 2006). In the sections below, we discuss the role of trehalose in the interactions of plants with bacterial and fungal pathogens, insects and parasitic plants, and consider the involvement of trehalose metabolism and signalling in plant interactions with symbiotic and other beneficial microbes. There are three aspects to the involvement of trehalose in pathogenic plant–microbe interactions: (i) trehalose metabolism in the bacterial or fungal pathogen, (ii) communication between the pathogen and the plant, and (iii) trehalose metabolism in the plant.

Most fungi and bacteria produce trehalose, and at least some plant pathogens depend on their trehalose metabolism for virulence. An example is *Magnaporthe oryzae*, the causal agent of rice blast disease. High turgor pressure builds up within a specialized structure, the appressorium, that the fungus employs to penetrate host plant tissue (Foster *et al.*, 2003). Deletion of the *TPS1* gene abolishes the capacity of the fungus to synthesize trehalose and weakens its pathogenicity, either by interfering with establishment of high turgor in the appressorium or with subsequent hyphal penetration (Wilson *et al.*, 2007). Following colonization of the host, fungal trehalase is implicated in further virulence-associated functions (Foster *et al.*, 2003; Fernandez and Wilson, 2012).

A second example is *Pseudomonas aeruginosa* strain PA14, a multi-host pathogen that infects nematodes, insects and vertebrates, as well as plants. *P. aeruginosa* mutants that lack the capacity to produce trehalose are unable to infect *A. thaliana*, but are unaffected in their ability to infect non-plant hosts (Djonović *et al.*, 2013). This strongly implicates trehalose as a plant-specific virulence factor in *P. aeruginosa*; however, it should be noted that alternative interpretations of the data were put forward in online comments regarding the paper by Djonović *et al.* (2013). Although the precise role of trehalose in plant infection by *P. aeruginosa* is not yet established, it appears to be necessary for the bacterium to take up nitrogen-containing nutrients and to replicate in the extracellular spaces of the plant.

Several studies suggest that extracellular trehalose may act as an elicitor of plant defence responses. Supplying trehalose exogenously to *A. thaliana* seedlings led to induction of pathogen defence-related genes, as well as changes in expression of stress-related transcription factors and genes linked to nitrogen metabolism (Bae *et al.*, 2005a,b; Aghdasi *et al.*, 2008). Spraying wheat plants with trehalose was found to induce resistance to powdery mildew (*Blumeria graminis* f. sp. *tritici*; Reignault *et al.*, 2001; Renard-Merlier *et al.*, 2007; Tayeh *et al.*, 2014). However, exogenous trehalose did not induce resistance to powdery mildew or late blight in tomato (Ishikawa *et al.*, 2005). These observations indicate that extracellular trehalose may be perceived by some, but not all, plants as a signal of pathogen attack, and thereby trigger pathogen defence responses. However, important questions remain unanswered. It is unclear how much trehalose leaks out from invading fungal and bacterial pathogens, and how significant pathogen-derived trehalose may be in triggering defence responses in comparison with well-characterized elicitors, such as flagellin.

Infection of *A. thaliana* with the clubroot pathogen, *Plasmodiophora brassica*, led to trehalose accumulation in infected organs (Brodmann *et al.*, 2002). As the accumulation of trehalose was accompanied by up-regulation of the *PbTPS1* gene, it was proposed that much of the accumulated trehalose was derived from the pathogen. Infection also led to induction of *AtTRE1* in roots and hypocotyls, and it was proposed this may represent a defence response to limit the accumulation of trehalose, which may otherwise adversely affect the plant's metabolism. Thus, AtTRE1 potentially has a dual role as a sensor of extracellular, pathogen-derived trehalose, and as a defence against excessive accumulation of trehalose (Gravot *et al.*, 2011).

Extracellular trehalose is potentially a sign of other dangers to the plant, including insects (Singh *et al.*, 2011), nematodes (Hofmann *et al.*, 2010) or parasitic plants such as *Cuscuta reflexa* (southern Asian dodder; Veluthambi *et al.*, 1981, 1982a,b). Aphid honeydew contains high levels of trehalose (Hodge *et al.*, 2013), potentially providing a signal of aphid attack. Infestation of *A. thaliana* with the peach potato aphid, *Myzus persicae*, led to systemic accumulation of trehalose in the plant that was dependent on aphid density (Hodge *et al.*, 2013). It has been proposed that TPS11 is the source of trehalose in *A. thaliana* and tomato plants infested with *M. persicae* (Singh *et al.*, 2011; Singh and Shah, 2012). However, there is no direct proof that AtTPS11 has enzymatic activity. Singh *et al.* (2011) concluded that AtTPS11 is a bifunctional TPS–TPP enzyme based on complementation of yeast *tps1Δ* and *tps2Δ* mutants, but used an unsuitable promoter for testing complementation (Vandesteene *et al.*, 2012), and their results conflict with those of a previous study in which an appropriate promoter was used (Ramon *et al.*, 2009), casting doubt on the reliability of the data reported by Singh *et al.* (2011).

The potential for trehalose to act as a signal of microbial pathogen attack is complicated by two factors. First, it may also be a signal of attack by insects or other herbivores, for which the plant needs to mount specific and different defence responses. Thus, the plant needs to distinguish between different potential sources of extracellular trehalose. Second, trehalose production is also a feature of many beneficial microbes, such as rhizobial and

mycorrhizal symbionts (Secks *et al.*, 1999; Streeter and Gomez, 2006; Rodríguez-Salazar *et al.*, 2009). Thus, any such defence responses must be suppressed in symbiotic and beneficial interactions.

It has long been known that trehalose accumulates in root nodules of legumes that form symbioses with *Rhizobium* spp. (Salminen and Streeter, 1986; Müller *et al.*, 1992, 2001; Farias-Rodriguez *et al.*, 1998; López *et al.*, 2008a,b; Domínguez-Ferreras *et al.*, 2009; Brechenmacher *et al.*, 2010). In rhizobial–legume symbioses, exogenous trehalose induced sucrose synthase and alkaline invertase activities, potentially increasing the supply of hexose sugars to the rhizobial symbiont (Müller *et al.*, 1998; Xie *et al.*, 2003; García *et al.*, 2005). The importance of trehalose in rhizobial symbioses was further demonstrated by manipulation of the trehalose biosynthetic capacity in *Rhizobium etli*. Inoculation of common bean (*Phaseolus vulgaris*) with a strain of *R. etli* engineered to over-express the *E. coli* TPS (otsA), and so produce more trehalose, led to formation of more nodules with higher nitrogenase activity and increased plant biomass compared to plants inoculated with a wild-type strain (Suárez *et al.*, 2008). Conversely, loss of the endogenous *otsA* gene from *R. etli* had a negative effect on nodule number, nitrogenase activity and plant biomass.

Many plants form symbioses with mycorrhizal fungi, which provide inorganic nutrients, e.g. phosphate, to the plant in return for carbohydrates. There is evidence that trehalose plays a significant role in ectomycorrhizal relationships (Rieger *et al.*, 1992; Corrêa *et al.*, 2010; Nehls *et al.*, 2010). Trehalose represents a major sink for carbon in *Amanita muscaria* and *Pisolithus microcarpus* ectomycorrhizae associated with poplar (*Populus tremula x tremuloides*) and *Eucalyptus globulus* roots, respectively (Martin *et al.*, 1998; López *et al.*, 2007). In *A. muscaria*, expression of fungal genes encoding enzymes of trehalose metabolism was induced upon formation of the ectomycorrhizal symbiosis (López *et al.*, 2008a,b), while in the *E. globulus*–*P. tinctorius* interaction, mycorrhizal colonization increased the allocation of carbon to trehalose in the mycelium (Martin *et al.*, 1998). In a further example, inoculation of grapevine with a non-symbiotic, plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN, led to up-regulation of trehalose metabolism in the plant and improved tolerance to chilling stress (Fernandez *et al.*, 2012).

In summary, trehalose is implicated as a virulence factor or signal molecule in the interactions between plants and a diverse array of other organisms, and these interactions may also have a significant effect on the plant's own trehalose metabolism. However, little is known about the underlying mechanisms and physiological significance of these responses. Understanding these interactions appears to be a fertile area for future research, potentially offering ways to improve crop plant defences against microbial pathogens and insect pests, while also promoting beneficial associations with symbiotic bacteria and fungi.

It is tantalizing to speculate on the changing function of trehalose metabolism and its relationship with sucrose metabolism during the evolution of plants. Trehalose may be a major storage and stress-protecting metabolite in primitive plants, and there is be significant competition for substrates between trehalose and sucrose biosynthesis. With the evolution of vascular tissues and adoption of sucrose as the main transport sugar, sucrose displaced trehalose from most of its original functions (Lunn and MacRae, 2003; MacRae and Lunn, 2012), freeing trehalose metabolism to take on new roles in sucrose signalling and responses to abiotic and biotic stresses. It is not too difficult to imagine how mechanisms for controlling the competition for substrates between sucrose and trehalose biosynthesis may have evolved to form the Tre6P–sucrose nexus of bi-directional control observed in higher plants. As the endogenous levels of trehalose dwindled, there may have been the opportunity for the plant to evolve sensing mechanisms by which exogenous or abnormally high trehalose levels are perceived as a sign of pathogen or herbivore attack and are used to trigger the appropriate defence responses.

FUTURE DIRECTIONS

It is clear that Tre6P and trehalose are essential for plant metabolism, development and growth, with increasing evidence for a role of Tre6P as a sucrose signal and for trehalose in stress responses. However, our understanding of this important signalling pathway is still rudimentary. To improve our understanding, the following research priorities may be defined. The first is to understand the molecular mechanisms by which sucrose regulates Tre6P levels. The second is to understand the function of the diverse families of class II TPS and TPP proteins by mutant analysis and study of trehalose metabolism in algae and non-vascular plants that have smaller *TPS* and *TPP* gene families. The third is to elucidate how trehalose formed by TPP activity is degraded, either by transport into the apoplast for degradation by extracellular trehalase or by the action of other currently undiscovered trehalose-metabolizing enzymes in the cytoplasm. The fourth is to better understand the modes of action of Tre6P and how sucrose signalling by Tre6P is integrated with other pathways of sucrose signalling, especially the bZIP11 pathway (Hummel *et al.*, 2009; Rahmani *et al.*, 2009; Ma *et al.*, 2011). This will probably require use of cell-specific and inducible expression systems, coupled with detailed analyses of metabolite levels, fluxes, protein phosphorylation patterns and transcript levels. The fifth is to define unambiguous Tre6P-dependent phenotypes that may be used for forward genetic screens. The sixth is to use genetic diversity to

obtain a broader understanding of the function of Tre6P, both within and between species, especially those that store and transport sugars other than sucrose and/or use different phloem-loading strategies (Fu *et al.*, 2011). Finally, the seventh is to deepen our molecular understanding of the role of trehalose in abiotic and biotic stress, and provide a rational basis for future engineering of trehalose metabolism to improve stress tolerance and pathogen defence in crop plants.

ACKNOWLEDGEMENTS

The authors' research is supported by a grant from the Fonds Wetenschappelijk Onderzoek (grant number G.0859.10 to P.V.D), the European Commission FP7 collaborative project TiMet (contract number 245142 to M.S.), the Bundesministerium für Bildung und Forschung (Plant KBBE-SAFQIM project 0315912, to J.L.) and the Max Planck Society (to M.S. and J.L.). Dedicated to Dr Marshall (Hal) Davidson Hatch and Prof. Hans-Walter Heldt on their 80th birthdays.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Effect of constitutive TPS and TPP expression on the relationship of Tre6P to sucrose.

Table S1. Meta-analysis of Tre6P and sucrose levels and Tre6P: sucrose ratios in wild-type plants.

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