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Trehalose Metabolites in Arabidopsis—elusive, active and central

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Trehalose is an alpha,alpha-1,1-linked glucose disaccharide. In plants, trehalose is synthesized in two steps. Firstly, trehalose-6-phosphate synthase (TPS) converts UDP-glucose and glucose-6-phosphate to trehalose-6-phosphate (T6P); secondly, T6P-phosphatase (TPP) converts T6P into trehalose and Pi. Trehalose is further cleaved into glucose by trehalase. In extracts of most plants, including Arabidopsis, levels of both trehalose and T6P are low, nearing detection limits, and this has delayed research into their function. Trehalose is transported widely in plants, but transport of T6P is not thought to occur except possibly at the subcellular level. Feeding trehalose to Arabidopsis seedlings alters carbon allocation with massive starch accumulation in cotyledons and leaves and absence of starch and growth in shoot and root apices.

The Arabidopsis genome has experienced extensive radiation of genes likely encoding enzymes of T6P metabolism: 4 and 10 genes are found with homology to TPS and TPP respectively and 7 genes are found with homology to both TPS and TPP. Complementation of *Saccharomyces cerevisiae* mutants has shown that AtTPS1, AtTPPA and AtTPPB are functional enzymes. In contrast just a single gene encoding a protein with trehalase activity has been found. Whilst most TPS proteins appear cytosolic, strikingly, some TPPs appear targeted to chloroplasts; trehalase on the other hand is extracellular. Transporters of trehalose and T6P have yet to be described. Arabidopsis *tps1* mutants are embryo lethal and results suggest that T6P is essential for several other steps in development including root growth and floral transition. Accordingly, altering T6P content has a profound effect on plant habitus and impacts metabolite profiles, sugar utilization and photosynthesis. These large effects have hindered dissection of cause and effect. In contrast, plants with large alterations in sucrose-6-phosphate concentrations are indistinguishable from wild type, suggesting very different functions for these compounds. Recently, T6P at low micromolar concentrations has been shown *in vitro* and *in vivo* to inhibit SnRK1 of the SNF1/AMPK group of protein kinases. This supports a function for T6P as a sugar signaling molecule integrating metabolism and development in plants in relation to carbon supply.

Genetic engineering of Arabidopsis as well as tobacco, potato and rice with TPS or TPS/TPP protein fusions reveals that trehalose metabolism also mediates multiple abiotic stress tolerances. Trehalose applications also mediate biotic stress resistances. Both *Escherichia coli* and *Saccharomyces cerevisiae* TPS/TPP protein fusions can be used to engineer stress tolerance suggesting that metabolites rather than proteins of the trehalose pathway are key stress tolerance elicitors. Results underscore the central role of trehalose metabolites in integrating carbon metabolism and stress responses with plant development.

LEVELS OF TREHALOSE METABOLITES IN ARABIDOPSIS ARE VERY LOW BUT FEEDING EXPERIMENTS UNCOVER SURPRISING EFFECTS

Trehalose is both a compatible solute and a carbon source in microbes

Trehalose is an alpha-1,1 glucose disaccharide lacking reducing ends which ensures chemical stability (Crowe, 2007). Its mass is indistinguishable from that of common disaccharides such as sucrose, maltose, cellobiose and laminaribiose. Trehalose is generally quantified and detected by LC-PAD or after derivatisation, using GC-FID or GC-MS (Muller et al., 2001; Roessner et al., 2000). NMR and specific trehalases have been used to confirm the identity of the compound.

Trehalose is a primary metabolite that may be synthesized through at least five pathways; only one pathway appears present in plants

Recognised trehalose biosynthesis pathways have been previously reviewed (Avonce et al., 2006; Elbein et al., 2003) and are presented in Figure 1. In summary, the TPS/TPP pathway converts glucose-6-phosphate (G6P) and UDP-glucose (UDPG) to

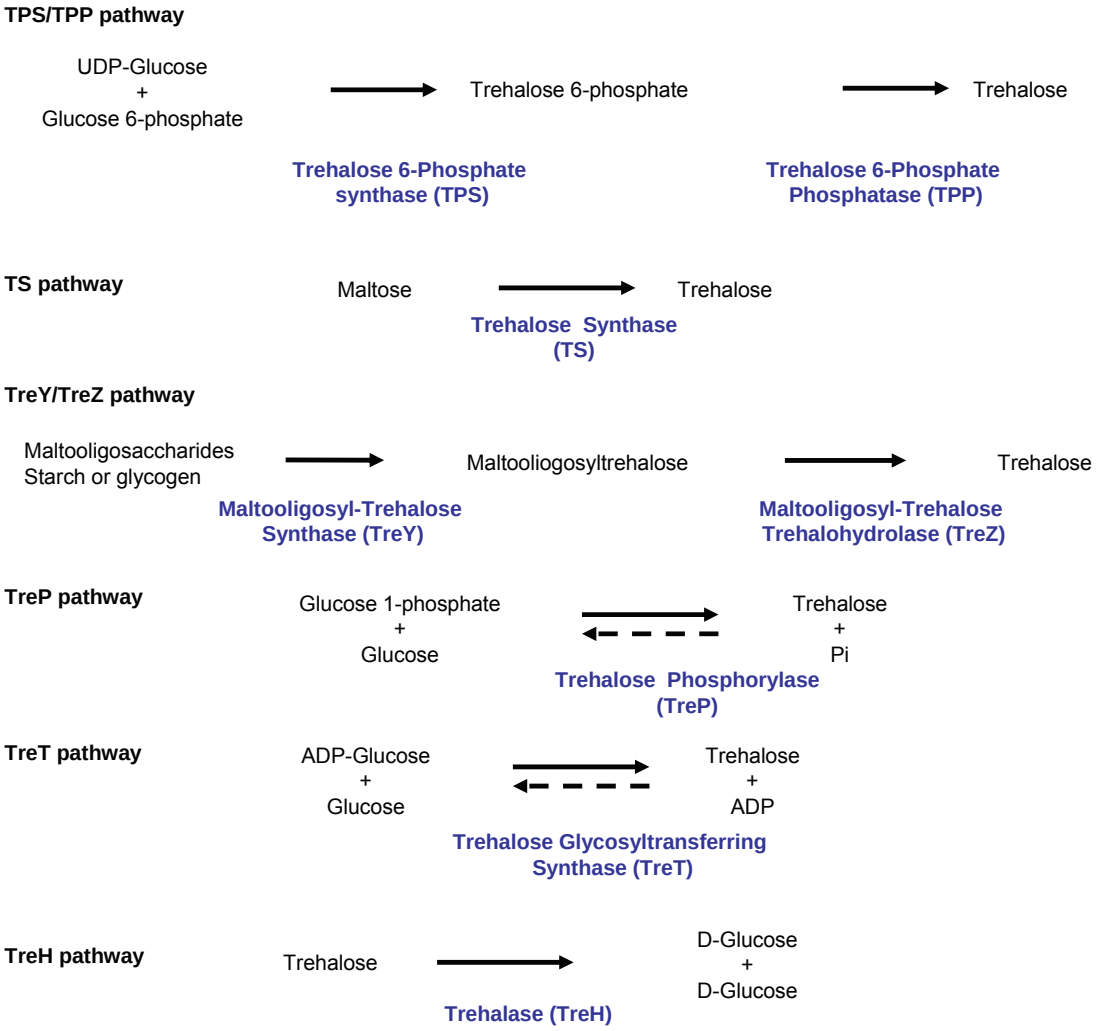

Figure 1. Known trehalose biosynthesis pathways. Enzymes are labeled in blue. The **TPS/TPP pathway** converts glucose-6-phosphate (G6P) and UDP-glucose (UDPG) to trehalose-6-phosphate (T6P) by trehalose-6-phosphate synthase (TPS). T6P is then cleaved to trehalose and inorganic phosphate by trehalose-6-phosphate phosphatase (TPP). In the **TreY/TreZ pathway** the terminal 1,6-linkage from a starch molecule is converted to an alpha 1,1 linkage by epimerization and then the terminal disaccharide is cleaved releasing trehalose. In the **TS pathway** maltose is converted to trehalose by trehalose synthase (TS). In the **TreP pathway** trehalose is synthesized from glucose-1-phosphate and glucose. Finally in the **TreT pathway** trehalose is synthesized from ADP-glucose (ADPG), or UDPG, and glucose. Trehalose is cleaved into two moieties of glucose by specific trehalases (**TreH pathway**). Plants have been shown to encode enzymes capable of trehalose synthesis via the TPS/TPP pathway (Blazquez et al., 1998; Chary et al., 2007; Pramanik and Imai, 2005; Satoh-Nagasawa et al., 2006; Shima et al., 2007; Vogel et al., 1998). Plants also contain genes that encode trehalase (Aeschbacher et al., 1999; Muller et al., 2001), but lack genes with homology to enzymes from the other pathways. Image reproduced from Avonce et al. (2006) with permission from BioMed Central.

trehalose-6-phosphate (T6P) by trehalose-6-phosphate synthase (TPS). T6P is then cleaved to trehalose and inorganic phosphate by trehalose-6-phosphate phosphatase (TPP). In the TS pathway maltose is converted to trehalose by trehalose synthase (TS). In the TreY/TreZ pathway the terminal 1,6-linkage from a starch molecule is converted to an alpha 1,1 linkage by epimerization and then the terminal disaccharide is cleaved releasing trehalose. In the TreP pathway trehalose is synthesized from glucose-1-phosphate and glucose. Finally, In the TreT pathway trehalose is synthesized from ADP-glucose (ADPG), or UDPG, and glucose. Trehalose is cleaved into two moieties of glucose by specific trehalases (TreH pathway). Plants have been shown to encode enzymes capable of trehalose synthesis via the TPS/TPP pathway (Blazquez et al., 1998; Chary et al., 2007; Pramanik and Imai, 2005; Satoh-Nagasawa et al., 2006; Shima et al., 2007; Vogel et al., 1998). Plants also contain genes that encode trehalase (Aeschbacher et al., 1999; Muller et al., 2001), but lack genes with homology to enzymes from the other pathways. This is consistent with observations from other eukaryotes where the TPS/TPP pathway is dominant, although some fungi have genes for the TreP pathway.

Trehalose metabolism is ancient and eukaryotes contain genes with both TPS and TPP domains

Genes encoding enzymes from trehalose metabolism are found almost ubiquitously (Avonce et al., 2006). Eubacteria have demonstrated all five possible pathways of trehalose biosynthesis whilst Archaea appear to lack the TS pathway. Several eubacterial species have multiple pathways. Eukaryotes have predominantly the TPS/TPP pathway. Invertebrates use trehalose as their blood sugar. Vertebrates are unable to synthesize trehalose but express trehalase particularly in the intestine. Common to the eukaryotic TPS/TPP pathway is the presence of enzymes with both TPS and TPP domains. *S. cerevisiae* ScTPS2 has a TPP function but also contains a TPS-like domain located N-terminally (De Virgilio et al., 1993). In contrast, Arabidopsis AtTPS1 has a TPS function but also contains a C-terminal TPP-like domain (Blazquez et al., 1998; Leyman et al., 2001). In fact all AtTPS1-11 contain two domains with homology to TPS (N-terminally) and TPP (C-terminally) suggesting that these enzymes are derived from a single event of gene fusion possibly inherited from the eukaryote before chloroplast endosymbiosis (Table 1). *S. cerevisiae* TPS/TPP occurs as a complex of proteins including the ScTPS1 and the ScTPS2 and it has been proposed that complex formation serves to control free levels of T6P in the cell (generally 100 μM) when trehalose is being synthesized (to above 1 M) for stress protection and as carbon reserve (Bell et al., 1998).

In Arabidopsis levels of trehalose metabolites are generally at current instrument detection limits

Trehalose in wild type plant extracts has been mostly quantified by GC-FID and GC-MS where it is near the detection limit ranging from 37 ng g^{-1} dry weight in whole Arabidopsis plants up to 100 ng g^{-1} dry weight in Arabidopsis flowers (Muller et al., 2001; Roessner et al., 2000; Suzuki et al., 2008). T6P inhibits some yeast hexokinases such as Yarrowia lipolytica hexokinase and this biochemical property has been exploited to determine T6P concentrations in plant extracts with levels in Arabidopsis seedlings at 4.5 nmol g^{-1} FW being reported (Blazquez et al., 1994; Pellny et al., 2004; Schluepmann et al., 2003). T6P has further been quantified in Arabidopsis seedlings using a procedure that includes a specific trehalase yielding 1.8 nmol g^{-1} FW (Schluepmann et al., 2004) and using LC-MS yielding a range of concentrations from 0.018 nmol g^{-1} FW in carbon starved to 0.482 nmol g^{-1} FW in seedlings with supplied carbon under low light conditions (Delatte et al., 2009; Lunn et al., 2006; Schluepmann et al., 2004). The LC/MS methods were adapted from a method detecting sucrose-6-phosphate in plant extracts (Chen et al., 2005) and are likely the most accurate applied to T6P quantification in plant extracts until now. Data support the view that T6P has a large dynamic range in plants consistent with a function as a sugar signaling molecule. These detection methods, however, do not distinguish between metabolites derived from plant or bacterial cells. Microbial symbionts can accumulate trehalose metabolites at concentrations two to three orders of magnitude higher than plants, so contamination could be significant. Further trehalose-containing metabolites have not been characterized in plant extracts, but little effort has been made towards this in part because of the contamination issue necessitating a genetic or cell biology approach.

T6P is a charged molecule unlikely to cross membranes without proteinaceous channels. Determination of the fine subcellular distribution of trehalose metabolism in Arabidopsis as well as the cell-specific expression of some genes encoding enzymes from trehalose metabolism will require precise methods in cell biology. *In vivo* staining using FRET nano-sensors as described for glucose and sucrose could be used but currently lacks the sensitivity required (Deuschle et al., 2006; Lager et al., 2006; Lalonde et al., 2005). Another alternative would be to convert trehalose or T6P into a molecule not present in plants and readily recognized by specific antibodies. A solution could be the conversion to trehalose mycolate using an enzyme from either *Mycobacteria* or *Corynebacteria* (Lederer, 1976). Trehalose mycolates are relatively insoluble and can be readily detected at very high sensitivity by antibodies. Enzymes that catalyse this conversion have not been characterized, however, and it is not known if they are sufficiently specific for their substrate and if they operate at sufficiently low concentrations of substrate. Finally trehalose metabolites are known to trigger transcriptional responses and this could be exploited in the same way as it was for the auxin-responsive promoter DR5 (Ulmasov et al., 1997).

Feeding trehalose metabolites to Arabidopsis uncovers surprising effects

Feeding trehalose to Arabidopsis elicits strong responses that are not generated by osmoticum controls such as sorbitol or mannitol. In seedlings grown on 25 mM trehalose, carbon allocation is reversed with a massive accumulation of starch from carbon fixed in the cotyledons and no starch accumulation in root tips (Wingler et al., 2000). When trehalose (100 mM) is supplied, seedlings germinate and extend cotyledons, but fail to develop primary leaves and the primary root does not grow beyond 2-5 mm (Schluepmann et al., 2004). Interestingly, the root meristem is reduced in size and cells in the extension zone swell and burst. Supply of metabolisable sugars in addition to 100 mM trehalose relieves the growth inhibitory effects of trehalose suggesting that starvation causes growth arrest; it further suggests that trehalose does not affect the ability of sink tissues to metabolise carbon allocated (Schluepmann et al., 2004). Starvation in the growth zones is not due to carbon sequestration as starch since seedlings of *pgm1* mutants unable to accumulate starch remain sensitive to external trehalose (Fritzius et al., 2001). Therefore trehalose may affect carbon transport or loading for transport. This effect could be at the subcellular level i.e. export from the chloroplasts to the cytosol. Alternatively the effect could be at the plant level i.e. interface between mesophyll and vascular bundles or simply involve transport inhibition in the phloem. Interestingly, trehalose competitively inhibits the sucrose-phosphate-phosphatase (SPP) of the cyanobacterium *Synechocystis* sp. (Fieulaine et al., 2007). Trehalose feeding could therefore potentially inhibit sucrose synthesis which would explain the strong phenotype that arises. Tobacco plants with antisense inhibition of SPP indeed accumulate starch in their leaves. They however have light-green leaves and do not display the dark green phenotype characteristic of plants grown on trehalose or accumulating T6P (Pellny et al., 2004; Schluepmann

Table 1. Summary table of the different Arabidopsis genes associated with trehalose metabolism together with AGI numbers, mutants, phenotypes, and expression patterns.

Name	AGI	Mutants	Phenotypes	References*	Gene-expression links
AtTPS1	AT1G78580	*tps1*	*tps1-1,* embryo lethal	Eastmond, et al. (2002), Baud, et al. (2006), Gomez, et al. (2006), Blasquez et al. (1998), Van Dijken et al. (2004), Schluepmann et al. (2003)	Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPS2	AT1G16980	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPS3	AT1G17000	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPS4	AT4G27550	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPS5	AT4G17770	*tps5*	Thermo-sensitive	Suzuki et al. (2008)	Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPS6	AT1G68020	*csp1****	Cell shape phenotype***	Chary et al. (2007)	Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPS7	AT1G06410	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPS8	AT1G70290	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPS9	AT1G23870	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPS10	AT1G60140	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPS11	AT2G18700	ND	ND	Heazlewood et al. (2003)	Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTRE1	At4g24040	ND	ND	Frison et al. (2007); Brodmann et al. (2002) Mueller et al. (2001)	Genevestigator Gene Atlas; Genevestigator Gene Chronologer Genevestigator Response Viewer
AtTPPA	AT5G51460	ND	ND	Vogel et al. (1998)	Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPPB	AT1G78090	ND**	ND**	Vogel et al. (1998)	Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPPC	AT1G22210	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPPD	AT1G35910	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPPE	AT2G22190	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPPF	AT4G12430	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPPG	AT4G22590	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPPH	AT4G39770	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPPI	AT5G10100	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer
AtTPPJ	AT5G65140	ND	ND		Genevestigator Gene Atlas; Genevestigator Gene Chronologer; Genevestigator Response Viewer

*For a comparison of the gene and protein sequences and discussions on phylogenetic relationships consult Leyman et al. (2001), Avonce et al. (2006) and Lunn (2007).

**RAMOSA3 encodes a TPP from maize affecting inflorescence structure. Because of multiple duplication events within or before the origin of the grasses, the closest Arabidopsis TPP homologue, TPPB, cannot be considered orthologous to either RAMOSA3 or the other member of the clyde, SRA, Satoh-Nagasawa et al. 2006.

***Needs verifying- refer to text section 2.5.2 Class II TPS.

et al., 2003; Schluepmann et al., 2004). Growth of Arabidopsis seedlings expressing T6P hydrolase on 100 mM trehalose supports the idea that T6P rather than trehalose causes growth arrest (Schluepmann et al., 2004). However, T6P in the presence of sucrose stimulates growth (Schluepmann et al., 2003), so these effects are dependent on the presence of high trehalose ie. a non metabolisable carbon source. Feeding 30 mM trehalose in the presence of 1% w/v sucrose induced accumulation of sucrose and starch in two-week-old Arabidopsis seedlings and induced proteins known from stress detoxification including AtGSTF2, AtDHAR1, AtSAMS2 and AtFQR1. It is thus possible that high trehalose released from endogenous pathogens functions as elicitor (Bae et al., 2005a). Early changes in transcription after feeding trehalose seem to support this hypothesis (Bae et al., 2005b). Repressed Rubisco activase, that is redox-activated by thioredoxin f similarly to ADPG pyrophosphorylase (AGPase), and reduced levels of ATP after 24 h trehalose feeding in the presence of 1% w/v sucrose (Bae et al., 2005a) are consistent with effects expected when T6P accumulates (Pellny et al., 2004; Schluepmann et al., 2003; Schluepmann et al., 2004).

Trehalose supplied externally is likely taken up by seedlings since expression of *E.coli* trehalase targeted to the cytosol allows thriving growth on trehalose medium due to glucose released from trehalose cleavage (Schluepmann et al., 2003; Schluepmann et al., 2004). Seedlings therefore do not have a sufficiently high activity of trehalase to detoxify trehalose supplied at 100 mM. This holds true for all Arabidopsis accessions tested thus far, with Cvi being the only accession with some low degree of resistance to 100 mM external trehalose (Aghdasi PhD Thesis, 2007). Feeding trehalose not only affects carbon allocation from carbon fixed by cotyledons, but also seedlings in the dark that respond to trehalose feeding. These seedlings lack hypocotyl elongation but still undergo skotomorphogenesis suggesting lack of resource for extension but sugar sensing and signal transduction (Aghdasi Utrecht University PhD thesis 2007; Wingler et al., 2000).

Feeding T6P has been attempted to rescue embryos lacking *AtTPS1* yet T6P appears to be not taken up by the embryos, probably because it is highly charged and therefore cell autonomous (Eastmond et al., 2002).

Validamycin A has been used as a specific competitive inhibitor of trehalase and its mode of action has been studied at the protein structure level with the *E.coli* periplasmic trehalase TreA (Gibson et al., 2007). Validamycin A is taken up by roots of tobacco and potato plants and readily transported to leaves (Goddijn et al., 1997). Feeding Validamycin A at 1 mM concentration leads to trehalose accumulation in tobacco and potato but only to levels of 0.021 mg g^{-1} FW suggesting that flux through the trehalase step in these species is low (Goddijn et al., 1997). Validamycin A, unlike trehalose does not cause reversal of carbon allocation in Arabidopsis seedlings when supplied at 10 mM (Wingler et al., 2000) and preparations (Duchefa Biochemie, The Netherlands) above 1 mM are toxic, inhibiting root development completely but not affecting starch accumulation in the cotyledons (Schluepmann et al. unpublished). Validamycin A at 10 μM has been used as a trehalase inhibitor combined with feeding trehalose at 25 mM to uncover effects of trehalose at mM concentrations (Ramon et al., 2007; Wingler et al., 2000). Validamycin A, however is not specific for trehalase and affects the activity of other enzymes of trehalose metabolism, for example, it also inhibits trehalose synthase and maltooligosyltrehalose synthase from *Bradyrhizobium japonicum* and *B. elkanii* (Streeter and Gomez, 2006). Validamycin A has therefore been considered as a trehalose mimic. Trehazolin on the contrary was shown to specifically inhibit trehalase from these *Bradyrhizobium* species not affecting high activities of trehalose synthase and maltooligosyltrehalose synthase in nodules (Streeter and Gomez, 2006).

Feeding trehalose dimycolate to Arabidopsis did not affect seedlings but we are unable to tell if the relatively insoluble mycolates are taken up from the medium (Schluepmann et al. unpublished). Trehalose dimycolates are synthesized by *Mycobacterium* and *Corynebacterium* species and their mode of action during *Mycobacterium* infection has been studied extensively in humans and other model mammals. Specific trehalose dimycolates elicit strong inflammatory responses and trehalose dimycolates are thought to be synthesized from T6P (Ryll et al., 2001; Takayama et al., 2005).

Conclusion: In Arabidopsis, trehalose is unlikely to function as a compatible solute or a carbohydrate reserve as in microbes because trehalose concentrations are very low. Research progress is hindered by the restricted ability to visualize and quantify trehalose metabolites *in planta,* but is now being achieved through a combination of genetics and analytical chemistry. Open questions remain about the subcellular distribution of the metabolites and about possible derivatives of T6P or trehalose. Feeding trehalose to Arabidopsis leads to reversion of sink/source relations suggesting that it may well be an interesting target for applications in plant breeding. To research this activity, increasingly refined annotation of the *Arabidopsis thaliana* genome provides knowledge of the genes encoding enzymes of trehalose metabolism. In addition, expression of these genes can now be followed at the cellular level and with very high detection sensitivity. Characterization of genes encoding enzymes of trehalose metabolism and transport has only just begun.

NOT ALL THE GENES IN THE TREHALOSE PATHWAY HAVE BEEN CHARACTERIZED YET GENETICS REVEAL AN ESSENTIAL FUNCTION FOR T6P

The Arabidopsis genome has undergone radiation in T6P metabolism

Enzymes of trehalose metabolism from Arabidopsis were initially cloned by complementation of *S. cerevisiae* trehalose pathway mutants. In this way *AtTPS1* was found to encode a TPS and *TPPA* and *TPPB*, TPPs (Blazquez et al., 1998; Vogel et al., 1998).

Homology searches in the complete Arabidopsis genome reveal a total of 11 TPS and 10 TPP homologues (Avonce et al., 2006; Leyman et al., 2001; Schluepmann et al., 2004). All of the TPS homologues also contain a TPP-like domain at the C-terminal of the protein whilst the TPP homologues are single domain proteins. Only one gene encoding trehalase is present, *AtTRE1* (Muller et al., 2001). Therefore radiation at the level of T6P rather than trehalose has occurred in Arabidopsis, and this holds true for other plants with sequenced genomes (Avonce et al., 2006; Lunn, 2007; Pramanik and Imai, 2005; Satoh-Nagasawa et al., 2006; Shima et al., 2007).

T6P metabolism is indispensable in Arabidopsis development

Surprisingly, Arabidopsis knock outs of *AtTPS1* are embryo lethal (Figure 2) suggesting that none of the other 10 TPS homologues can replace its function (Eastmond et al., 2002). It also suggests that trehalose metabolism is critical at that stage of development. Complementation of *tps1* with inducible *AtTPS1* shows that AtTPS1 is also required for root growth and transition from vegetative to floral development (van Dijken et al., 2004). *tps1* can be complemented with *E. coli* TPS showing that T6P is required at embryo as well as at the other stages of development (Schluepmann et al., 2003). Yet, *AtTPS2-6* are apparently most highly expressed in embryo and seed (Avonce et al., 2006 from Genevestigator) and given they cannot substitute for TPS1 in *tps1* mutants, probably do not contribute to T6P synthesis in these tissues. We are therefore left wondering if the roles of these gene products are regulatory rather than biosynthetic, if they are present in different subcellular compartments to AtTPS1 or if they perform a different biosynthetic reaction altogether. A regulatory role is also presumed for the *S. cerevisiae* ScTPS3 and ScTSL1 proteins that have seemingly no biosynthetic activity but contain both domains with homology to TPS and TPP (Bell et al., 1998). ScTPS3 and ScTSL1 appear to stabilize the TPS/TPP enzyme complex that occurs in *S. cerevisiae.*

Gene sequences and protein targeting predict a wide subcellular distribution of trehalose metabolism

Trehalase from *Glycine max* is an apoplastic glycoprotein whereas trehalase from Arabidopsis appears to be plasma membrane bound with the catalytic domain on the apoplastic side of the membrane (Aeschbacher et al., 1999; Frison et al., 2007). Localization of AtTPS1 is cytosolic as determined by a combination of immunogold labeling in TEM and translational fusions with GFP (Geelen et al., 2007). AtTPS11 was found in mitochondrial fractions as identified by LCMS/MS (Heazlewood et al., 2004). In addition, several AtTPP, AtTPPA (At5g51460), AtTPPG (At4g22590) and AtTPPI (At5g10100) have a predicted subcellular location in the chloroplast (Emanuelsson et al., 2007).

The data thus infer that trehalose is broken down only after export to the apoplast. Trehalose is likely synthesized in the cytosol by a combination of TPS and TPP activities or by a bifunctional enzyme such as AtTPS6, although catalytic activity of this enzyme requires confirmation. T6P is likely generated in the cytosol and possibly transported to the chloroplasts where it then could be cleaved to trehalose by the TPP enzymes that appear likely targeted to the chloroplast. Further research is required to study the subcellular localization of enzymes from trehalose metabolism and to identify transport molecules. Such wide subcellular distribution suggests central functions, possibly including organellar feedback regulation. Indeed, T6P controls redox-regulation of AGPase, the rate-limiting enzyme of starch synthesis in chloroplasts. Because it is assumed that T6P is synthesized in the cytosol, T6P may convey information from the cytosol to regulate chloroplastic functions, such as starch synthesis. Homology searches in the complete Arabidopsis genome reveal a total of 11 TPS and 10 TPP homologs (Kolbe et al., 2005; Table 1).

Protein structures and possible functions of different genes annotated as part of trehalose metabolism in Arabidopsis

Analysis of genetic drift amongst the numerous homologues of TPS and TPP suggests that evolutionary selection retains the specificity of each of these genes: mutations are not random and maintain synonymous codons over the entire sequence of TPS and over almost all of TPP except for five codons (Avonce et al., 2006). Therefore TPS and TPP homologues must each fulfill a specific function required by selection.

Closer examination of *AtTPS1-11* allowed classification of these genes into two classes according to their *TPP* sequence (Leyman et al., 2001). Class I TPS (*AtTPS1-4*) have lost the typical phosphatase boxes in the C-terminal TPP domain. Class II TPS (*TPS5-11*) retained the typical phosphatase boxes in the C-terminal TPP domain. Figure 3 presents an alignment of the 11 Arabidopsis TPS proteins with *E.coli* OtsA (TPS), OtsB (TPP) and *Thermoplasma acidophilum* TPP (TaTPP). The three-dimensional structures of OtsA (Gibson et al., 2002a; Gibson et al., 2002b; Gibson et al., 2004) and of TaTPP (Rao et al., 2006) are known and amino acids have been identified that are critical for the reaction with substrate. Structures from bacterial enzymes have also been superimposed onto plant sequences to predict activity of the domains from the plant proteins (Lunn, 2007).

Class I TPSs are large proteins more than 1000 amino acids long with both TPS and TPP domains where the TPP domain appears to no longer have functional phosphatase boxes (Leyman et al., 2001; Van Dijck et al., 2002; Figure 3). Sucrose phosphate synthases similarly have a non functional SPP domain which was proposed to serve the purpose of substrate channeling (Fieulaine et al., 2005), but in the case of TPS it could also serve to sequester T6P. The AtTPS1 enzyme is special because of an N-terminal extension, which is not found in other Arabidopsis TPSs and was shown to mediate regulation of TPS activity; it is also found in the SlTPS1 from *Selaginella lepidophylla*, a desert resurrection plant that accumulates large amounts of trehalose (Van Dijck et al., 2002). A study of proteins interacting with AtTPS1 reveals that the N-terminal peptide in AtTPS1 appears to interact with KCA, a putative microtubule motor (Geelen et al., 2007). AtTPS1 also has a unique small low complexity region at the carboxy terminus (Figure 3). *tps1* can be complemented by *E. coli* OtsA. The likely *in planta* function of AtTPS1 is thus synthesis of T6P (Schluepmann et al., 2003). *AtTPS1* homologues have been found in every plant species thus far analysed including banana, poplar, rice, maize and sorghum. Class I *TPS1* homologues were also found in prasinophyte algae thought to represent a primitive lineage of land plants. These homologues generally constitute the single Class I TPS gene except in the species of the Brassicaceae and in the moss *P. patens* (Lunn, 2007). Arabidopsis *AtTPS2* and *4* are expressed in developing seeds but their function remains unknown. *AtTPS3* is not represented in EST data and it is not detected by micro-array expression profiling. The AtTPS3 protein moreover appears to be deleted in a part of its TPP domain (Lunn, 2007 and Figure 3).

Class II TPSs are equally as large as those from Class I; the proteins contain both a TPS and a TPP domain, but the phosphatase boxes have been retained in the TPP domain (Leyman et al., 2001; Figure 3). Recombinant AtTPS5 failed to exhibit activity *in vitro* (Harthill et al., 2006). AtTPS5 interacts with the transcriptional co-activator MBF1c *in vitro* in *S. cerevisiae* two-hybrid assays

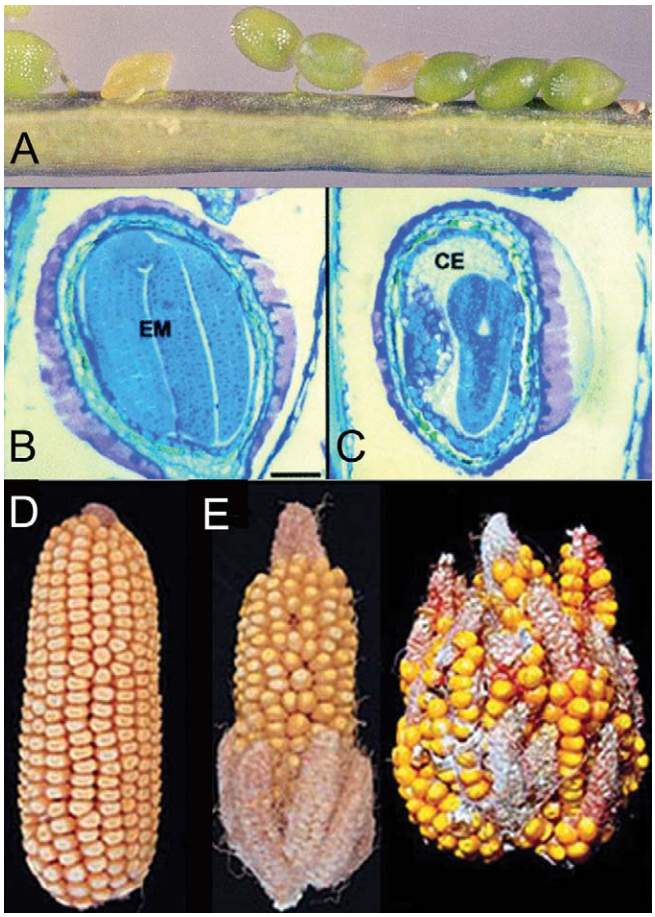

Figure 2. Mutants reveal that trehalose metabolism is involved at key steps in development in Arabidopsis and corn.
A) Silique from a tps1/TPS1 heterozygous Arabidopsis plant where 1 in 4 seeds with tps1/tps1 genotype do not contain the mature embryo but instead an embryo seemingly arrested at torpedo stage. Photo courtesy A. van Dijken.
B) Toluidine blue stained cross section of wild-type Arabidopsis seed with embryo at the mature stage; EM, embryo. Scale bar is 100 µm. Image reproduced from Eastmond et al. (2002) with permission from Blackwell Publishing.
C) Toluidine blue stained cross section of tps1 mutant seed with embryo arrested at torpedo stage; CE, cellular endosperm. Image reproduced from Eastmond et al. (2002) with permission from Blackwell Publishing.
D) Architecture of wild type B73 corn female inflorescence, the ear, after fertilization when seed is ripe. Image reproduced with permission from Macmillan Publishers Ltd: Nature, Satoh-Nagasawa et al. (2006).
E) Architecture of ramosa3 (ra3) mutant ears in B73 background (left) and in mixed genetic background (right) where axillary meristems lose their determinancy and change identity leading to abnormal branches at the base of the ear. ra3 alleles have lesions in a TPP belonging to a clade specifically found in grasses. Image reproduced with permission from Macmillan Publishers Ltd: Nature, Satoh-Nagasawa et al. (2006).

and is thought to be required for basal thermo-tolerance of Arabidopsis (Suzuki et al., 2008). *AtTPS6* was reported to complement *S. cerevisiae* strains deficient in *ScTPS1* growing on glucose and *S. cerevisiae* strains deficient in *ScTPS2* growing at 38°C (Chary et al., 2007) but this result required further confirmation. Phylogenetic trees constructed with Class II TPS from Arabidopsis, poplar and rice support 5 orthologous groups each including *AtTPS5*, *AtTPS6*, *AtTPS7*, *AtTPS8-10* and *AtTPS11* (Lunn, 2007).

AtTPS5, 6 and 7 are phosphorylated and bind 14-3-3 proteins (Harthill et al., 2006). 2-deoxy glucose feeding increases extractable SnRK1 activity and stimulates Th49 phosphorylation of AtTPS5 in Arabidopsis cells. Authors suggest that AtTPS5 may be a target of 14-3-3 binding after SnRK1 phosphorylation but only show *in vitro* phosphorylation of Thr49 and Ser22 from AtTPS5 by partially purified plant SnRK1. This is particularly interesting for AtTPS5 as its expression is very responsive to sucrose (Schluepmann et al., 2004). In addition, Class II AtTPS8-11 contain SnRK1 target sequences that are phosphorylated *in vitro* by Arabidopsis extracts in the absence of Ca^{2+} as would be expected by SnRK1 protein kinase (Glinski and Weckwerth, 2005). We thus conclude that Class II TPS likely are regulated by known nutrient signal transduction integrators such as SnRK1, but the significance and impact of this regulation on the function of the pathway is not clear.

TPP enzymes are single domain proteins and contain the conserved phosphatase boxes (Thaller et al., 1998; Vogel et al., 1998). AtTPPA and B complement *S. cerevisiae* defective in *ScTPS2*; crude extracts from these complemented *S. cerevisiae* make trehalose from T6P but the specific activities obtained were 5-12 times lower than with ScTPS2. The activities were specific for T6P and did not cleave sucrose-6-phosphate or G6P. In rice, OsTPP1 and OsTPP2 have been characterized biochemically: recombinant enzymes showed a high specificity for T6P and worked at higher affinity with 10 fold lower K_m compared to microbial enzymes (Pramanik and Imai, 2005; Shima et al., 2007). Maize RA3 (encoded by *RAMOSA3*) is a TPP shown to complement *S. cerevisiae* defective in *ScTPS2* and its recombinant protein also cleaves T6P specifically (Satoh-Nagasawa et al., 2006). RA3 is most similar to AtTPPB and AtTPPC but the many gene duplications that occurred before and after evolution of grasses may not necessarily mean that these genes have orthologous functions. Grasses, however, do have a RA3-specific TPP clade. RA3 is 361 amino acids long and has an 80 amino acid N-terminal sequence that is not conserved amongst other TPPs followed by the TPP domain. *ra3* mutants are on the same genetic pathway as *RA1* and *ra3* mutants have changes in *RA1* expression whilst *ra1* mutants are unaffected in *RA3* expression. Results therefore suggest that RA3 is upstream of RA1 in the RAMOSA pathway. RA1 is a transcription factor and authors speculate that RA3 could in addition to its enzymic function also function as a regulator of transcription to explain the developmental alterations in *ra3* mutants (Satoh-Nagasawa et al., 2006). Phylogenetic analyses of plant TPP sequences reveal that they are most closely related to proteobacteria and thus likely originate from the endosymbiotic ancestor of mitochondria (Lunn, 2007).

Transporters of trehalose or T6P are not yet characterized in plants. From insects, *TRET1,* a specific trehalose transporter that facilitates transport across membranes has been recently described (Kikawada et al., 2007). The TRET1 protein sequence is homologous to proteins from Arabidopsis encoded by At1g75220, At1g19450 and by ERD6, a sugar transporter induced by dehydration and cold treatment (Kiyosue et al., 1998). At1g75220 and At1g19450 are likely targeted through the secretory pathway to

Figure 3. Alignment of Arabidopsis TPS proteins with *E.coli* OtsA (TPS), OtsB (TPP) and *Thermoplasma acidophilum* TPP (TaTPP).

the plasma membrane or the endomembrane system. They both belong to the monosaccharide/sugar alcohol/polyol transporter superfamily but have not been functionally characterized. Presence of a transporter for import of T6P across the chloroplastic envelope has been inferred (Smith and Stitt, 2007). T6P is unlikely to be transported by a member of the above-mentioned gene family. A member of the plastidic phosphate translocator family including TPT, GPT, XPT, or PPT could be involved. GPT was demonstrated to transport G6P, but it was not tested for T6P transport (Kammerer et al., 1998; Niewiadomski et al., 2005). There are two related GPT genes in Arabidopsis, At5g54800 (*GPT1*) and At1g61800 (*GPT2*). A knockout of *GPT1* is lethal, whereas a knockout in *GPT2* is indistinguishable from wild type (Niewiadomski et al., 2005). A better understanding of this aspect of trehalose metabolism will be essential for elucidation of the mechanism of the role it plays in plants.

Expression of the genes associated with Arabidopsis trehalose metabolism is tightly controlled

Small changes in T6P levels bring about large changes in plant metabolism and development as seen by manipulation using *E. coli* enzymes and by changes in the levels of endogenous enzymes (Figure 4). Therefore, tight control of genes involved in T6P metabolism is likely to be paramount.

TPS and *TPP* genes respond strongly and rapidly to a number of important hormones and nutrients. *TPS8* is induced by cytokinin (Brenner et al., 2005). Two *TPSs* (*TPS9* and *TPS10*) and four *TPP* genes (*TPPB*, in particular) are regulated by nitrate (Wang et al., 2003). Rice *TPP* genes are induced by ABA (Pramanik and Imai, 2005), as is *TPS1* in guard cells (Leonhardt et al., 2004).

Interestingly, in contrast to sucrose-induced *TPS5*, *TPS8* is strongly dark induced (Piippo et al., 2006). Starvation treatments induced by low sugar and in the starch-less mutant *pgm1* induced several *TPSs,* but repressed a *TPP* (Thimm et al., 2004). Hypoxia, another form of starvation, increased expression of four genes associated with trehalose metabolism (*TPPA*, *TPPB*, *TPPJ* and trehalase) and repressed *TPS11* (Liu et al., 2005). The significance of the induction of these genes and their relationship with T6P levels is not yet known. If starvation and high sugar are both capable of inducing T6P synthesis, for example, then this would imply more than one T6P target, or the same target but in different tissues with opposing function such as source and sink. A *TPP* from corn, *RAMOSA*3, for example is expressed in a very restricted cell-file and preliminary results suggest that some *TPSs* from Arabidopsis have very restricted expression patterns (Satoh-Nagasawa et al., 2006). This contrasts with the expression patterns of *AtTPS1* and *AtTPS6* that are expressed ubiquitously at all stages of development (Chary et al., 2007; van Dijken et al., 2004). Analysis of the expression of all genes associated with trehalose metabolism in Arabidopsis roots using a dataset

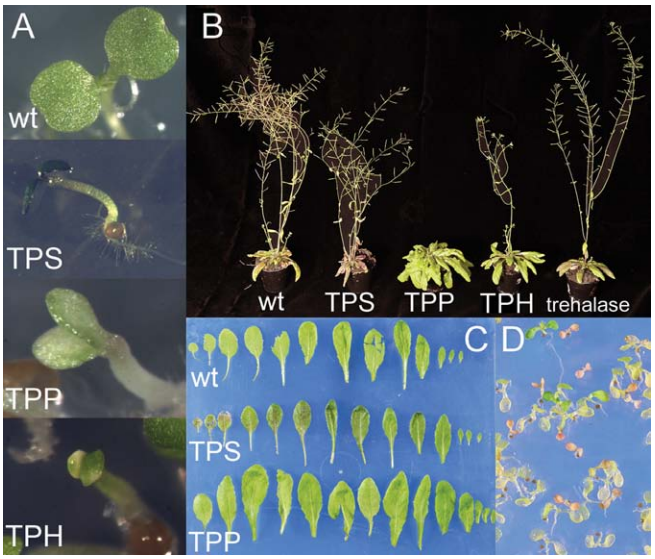

Figure 4. Phenotypic changes typically observed when altering T6P steady states. Constitutive over-expression of *E.coli* TPS in Arabidopsis results in a two fold increase in T6P steady state levels, seedlings with dark green cotyledons (**A**, **TPS**) and small bushy plants with dark green and smaller leaves compared to wild type (**B** and **C**, **TPS**). Constitutive over-expression of *E.coli* TPP (converts T6P to trehalose) or expression of *E.coli* trehalose-phosphate-hydrolase (**TPH**) (converts T6P to glucose-6-phosphate and glucose) results in up to 3 fold reduced T6P steady state levels in Arabidopsis. In contrast to plants with high T6P levels, plants with low T6P levels have partially bleached cotyledons at seedling stage (**A**, **TPP** and **TPH**) and pale green leaves that are larger than wild type at rosette stage (**B** and **C**, **TPP** and **TPH**). Expression of *E.coli* trehalase yields Arabidopsis seedlings able to thrive on medium with 100 mM trehalose compared to wild type seedlings that cease growing on this medium (**D**). Arabidopsis expressing *E.coli* trehalase, however, have levels of T6P and a phenotype indistinguishable from wild type (**B**, **trehalase**). Image reproduced from Schluepmann et al. (2003) with permission from PNAS.

generated from cell-sorted root protoplasts is shown in Figure 5 (Birnbaum et al., 2003; Brady et al., 2007). *TPS1* transcript is enriched in cells from the stele, in particular in the maturation stage and after full differentiation, *TPS8* is found highly expressed in stem cells including quiescent center and initials. *TPS11* is found expressed in the root tip and is also very abundantly expressed in the stem cells. Expression of some *TPP* genes is also highly specific: *TPPD*, for example is seemingly only expressed in the columella, whilst *TPPB* is mostly expressed in the stele of mature zones. *TPPG* is the most highly expressed gene and ubiquitously expressed with highest levels found in stage 3 maturation zone tissues. Specific cell files therefore express a specific set of genes from trehalose metabolism. In the columella, for exam-

Figure 3. (continued)

Alignment was using the full-length protein sequences with MAFFT (Katoh et al., 2005; Katoh and Toh, 2008) and the figure was made using the Jalview alignment editor (Clamp et al., 2004). Red squares indicate amino acids interacting with substrates at the TPS active site of crystals from OtsA (Gibson et al., 2002b; Gibson et al., 2004), red stars indicate amino acids interacting with substrates at the active site of crystals from TaTPP (Rao et al., 2006). Red boxes mark the conserved phosphatase boxes.

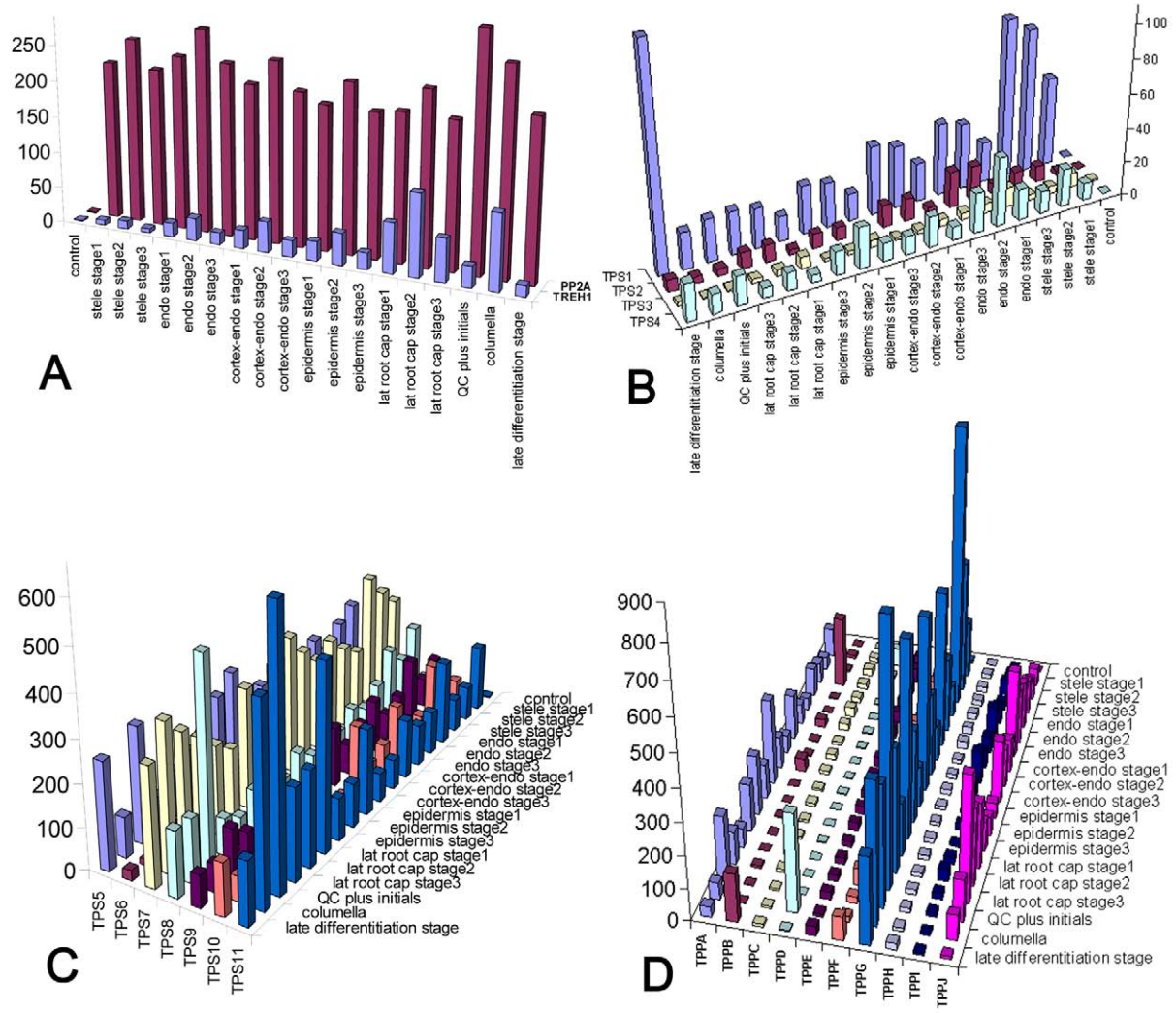

Figure 5. Expression of genes associated with trehalose metabolism in protoplasts of different root tissues. Data are from (Birnbaum et al., 2003; Brady et al., 2007); tissue identities are defined in these references.

A) Expression of the reference gene *PP2A* (At1g13320) did not significantly vary amongst the different tissues, whilst *TRE1* encoding trehalase is mostly expressed in lateral root cap and columella at 3 fold less than *PP2A*. For all genes there was no difference between the sum of gene expression over all tissues and the gene expression extracted from root before protoplast release suggesting that the procedure of cell wall breakdown and cell sorting did not alter expression of the genes (control).

B) Expression of Class I *TPS* is generally low with *TPS1* being the most highly expressed

C) In root tips, expression of Class II *TPS* is generally higher than Class I *TPS* except for *TPS6*; *TPS* Class II are expressed in all tissues concomitantly yet their relative expression varies: expression of *TPS8* is particularly high in the quiescent center for example

D) Expression levels of TPP genes differ amongst each other and appear in some cases to be extremely tissue specific – e.g. *TPPD* (columella) and *TPPB* (mostly stele). *TPPG* expression is ubiquitous and very high in the maturing root zone.

ple, all *TPS* genes except *TPS3* appear expressed as well as *TPPA, D, G* and *J*. Analysis of trehalose gene expression in roots therefore suggests that many *TPS* genes are simultaneously expressed and therefore may have differing roles in individual cells. Expression of the complement of *TPP* genes, however, appears to be more specific.

In addition to transcriptional regulation, regulation of the translation of TPPF and G by a specific conserved upstream open reading frame is likely (Hayden and Jorgensen, 2007).

Post-translational modification of the Class II TPSs by phosphorylation can regulate their activity and their binding to 14-3-3 proteins (Harthill et al., 2006). Multisite phosphorylation of Class II TPSs by both SnRK1 and Ca^{2+}-dependent protein kinases seems probable (as with sucrose phosphate synthases), giving the possibility of enzyme activation/inactivation in response to different internal and environmental cues (Paul, 2007).

GENETICS IMPLY TREHALOSE METABOLISM IN THE INTEGRATION OF METABOLISM AND DEVELOPMENT

Mutants in genes from the trehalose pathway have developmental defects

Plant development can be seen as a set program of events in which metabolic and developmental pathways draw on common resource pools, for example UDPG for cell wall synthesis during cell division and expansion. The maize *RAMOSA3* (RA3) gene, however, confirms a role for a gene of the trehalose pathway in controlling inflorescence branching in maize (Satoh-Nagasawa et al., 2006; Figure 2). RA3 encodes a TPP and is expressed in discrete domains subtending axillary inflorescence meristems. RA3 functions through the predicted transcriptional regulator RAMOSA1 and research has yet to reveal if and how TPP activity of RA3 and RAMOSA1 are linked.

The embryo-lethal phenotype of *tps1* mutants suggests a role of AtTPS1-mediated T6P production during embryo development. Eastmond et al. (2002) showed that *tps1* embryos are sensitive to externally supplied sucrose. These results are consistent with sucrose sensitivity of engineered plants expressing either TPP or TPH that contain low levels of T6P (Schluepmann et al., 2003) suggesting that T6P is required for carbon utilization and growth. Further consistent with the sucrose sensitivity of plants with low T6P is the free sugar accumulation in *tps1* mutant embryos (Gomez et al., 2006). In contrast, seedlings with higher steady state levels of T6P than wild type grow faster on medium with metabolisable sugars. Seedlings with high T6P levels compared to wild type also have low levels of free sugars and accumulate starch (Schluepmann et al., 2003). Detailed analysis of developing *tps1* embryos reveals that these embryos develop at a much slower pace compared to wild type (Eastmond et al., 2002; Gomez et al., 2005; Gomez et al., 2006) and that development beyond torpedo stage does occur after cold stratification and incubation on agar medium for periods ranging from 1-3 weeks (Gomez et al., 2006). Furthermore, torpedo stage *tps1* embryos displayed an expression profile unlike torpedo stage wild type embryos but much resembling the profile of mature wild type embryos (Gomez et al., 2006). *tps1* embryos therefore do undergo the procession of gene expression patterns typically associated with the progression through the embryo development stages. Cellular differentiation also occurs according to wild type embryo patterns in the *tps1* embryos with oil and protein bodies accumulating in *tps1* embryos seemingly arrested at torpedo stage; these bodies are typically found in wild type embryos at maturation stages including bent cotyledon stage. *tps1* embryos therefore are not affected in programs controlling metabolic and cellular development. Overall accumulation of protein and oil end products is reduced. Interestingly, *tps1* embryos have thicker cell walls compared to wild type embryos at either torpedo or bent cotyledon stage. These walls contain an epitope not found in wild type embryos and recognized by the LMC2 monoclonal antibody previously described as recognizing arabinogalactan proteins found at the plasma membrane of root apices of a number of plant species (Gomez et al., 2006; Smallwood et al., 1996). However, a number of cell wall related transcripts were down regulated. Interestingly the pectin fraction of the cell walls was increased. This represents a major flux of carbon in Arabidopsis. Cell wall thickening may be a consequence of reduced synthesis of oil and protein and reduced growth. It is possible that cell wall biosynthesis is a direct target of T6P. T6P is made from G6P and UDPG; UDPG is a known precursor of cell wall components. Incidentally cotton cellulose synthase CESA1 is redox-regulated by thioredoxin as AGPase but in the opposite direction (Doblin et al., 2002; Kurek et al., 2002). Expression analysis of the CycB1::GFP fusion in *tps1* revealed that the rate of cell division is much reduced in *tps1* embryos (Gomez et al., 2006). It is not known how the absence of T6P synthesis influences cell division in *tps1* embryos. The strong phenotype of these embryos makes it difficult to separate primary effects of T6P from further downstream effects such as accumulation of sucrose and starch.

Nevertheless, taken together the data point to an important role of trehalose metabolism in development, but not in the coordination of the succession of developmental events, rather more likely in processes of cell division.

T6P links photosynthesis and development

Tobacco and Arabidopsis plants with high T6P levels have high photosynthetic capacity per unit leaf area due to more Rubisco and chlorophyll (Pellny et al., 2004). This striking effect of perturbing T6P steady state in plants probably reflects the complex nature of the regulation of photosynthesis, which is integrated at the whole plant level and with the production of leaf area. Low T6P levels produced the opposite phenotype of lower photosynthetic capacity per unit leaf area, but paradoxically more productive plants, as leaf area and photosynthesis per plant were greater. It is likely that T6P affects early leaf development which affects leaf size and investment of photosynthetic apparatus. It is unclear if the effect on photosynthesis is linked to the impact of T6P on starch synthesis (Kolbe et al., 2005). Altered T6P content does not appear to alter NADPH/ NADP ratios. It is proposed that the impact of T6P on the redox regulation of AGPase is separate from effects on photosynthesis.

Does T6P have a metabolic function or a signaling role?

Hypotheses have been that T6P could be required as substrate for an essential reaction in metabolism or it could be required as a signaling molecule sensed and then transduced into signaling cascades. Metabolite profiling of *tps1* mutant embryos or *tps1* seedlings after genetic rescue of the embryos with inducible *AtTPS1* revealed vast changes in metabolite steady states when compared to wild type with most changes in sucrose, fructose and glucose (Gomez et al., 2006; Schluepmann et al., unpublished). In addition, G6P levels in seedlings expressing *E. coli* TPS or TPP are decreased and increased respectively (Pellny et al., 2004; Schluepmann et al., 2003). This is similar to *S. cerevisiae* deleted in *ScTPS1,* where T6P was shown to inhibit hexokinase and so low T6P results in high G6P steady states (Blazquez et al., 1993). *S. cerevisiae* without T6P is unable to grow on rapidly fermenting sugars such as glucose and it has been suggested that this is due to de-regulation of glycolysis with hyper-accumulation of sugar phosphates and depletion of ATP (Thevelein and Hohmann,

1995). Hexokinases from plant extracts, however, are not sensitive to T6P (Eastmond et al., 2002). Depletion of ATP due to insufficient phosphate recycling is unlikely since plants with 1000-fold accumulation of sucrose-6-phosphate do not have obvious signs of phosphate limitation (Chen et al., 2005). The main arguments for T6P as a signaling molecule are its low concentration in cells, wide dynamic range combined with the large changes in metabolism and morphology in plants that arise as a consequence of relatively minor changes in the steady state levels of T6P (Figure 4). Such changes are not found when changing the steady states of the similar molecule sucrose-6-phosphate, the precursor of sucrose or the regulatory molecule fructose-2,6-bisphosphate (Chen et al., 2005; Nielsen and Stitt, 2001). We know that T6P directly or indirectly affects specific redox regulation processes in the chloroplasts such as for example the redox activation of AGPase; the effect is independent of the NADPH/NADP ratios. The effect is found in mature leaves of Arabidopsis as well as in potato tubers. T6P mediated redox-activation is required for increased starch deposition when sugars are available (Kolbe et al., 2005). In addition, sugar-induced increases in T6P correlate with redox-activation of AGPase and increased rates of starch synthesis (Lunn et al., 2006). However most of the phenotypes produced where the pathway has been genetically modified in mutant and transgenic lines cannot be explained simply in terms of the effect on starch metabolism but rather support a more central function.

T6P inhibits SnRK1 of the SNF1/AMPK group of protein kinases

Recently, T6P was reported to inhibit SnRK1 (AKIN10/ AKIN11) of the family of calcium-independent Ser/Thr protein kinases that include AMPK of mammals and SNF1 of yeast (Zhang et al., 2009). These conserved protein kinases perform a fundamental role in transcriptional, metabolic and developmental regulation in response to energy limitation and starvation of carbon source (Hardie 2007). SnRK1 consists of a heterotrimeric complex of a catalytic alpha subunit together with beta and gamma regulatory subunits, plus a number of known and likely unknown interacting and regulatory factors (Polge and Thomas 2007). It was shown that low micromolar concentrations of T6P inhibit SnRK1 activity at a site on the SnRK1 complex distinct and physically separable from the catalytic site via an unknown intermediary factor. T6P inhibited SnRK1 most strongly in seedlings and young actively growing tissues. Inhibition was also found in all other plant tissues tested except in mature leaves that lacked the intermediary factor. Striking confirmation of the effects of T6P on SnRK1 activity measured using plant extracts was made by comparing effects on gene expression in *A. thaliana* with altered T6P compared to plants with altered AKIN10 expression. Baena-Gonzalez et al. (2007) using a transient protoplast expression system established a 1000 or so genes that were directly regulated by SnRK1. Using seedlings Zhang et al. (2009) found that genes normally induced by SnRK1 were repressed by T6P and those normally repressed by SnRK1 were induced, strongly supporting a role for T6P as inhibitor of SnRK1 *in vivo*. Overall there was a strong upregulation of biosynthetic pathways by T6P. It had previously been found that T6P promotes growth of seedlings on sucrose (Schluepmann et al. 2003). These data explain this finding and support a role for T6P as promoter of biosynthetic reactions in growing tissues through inhibition of SnRK1. It is likely that a major function of SnRK1 in plants, as for SNF1 and AMPK in other organisms, is to activate catabolic processes under starvation stress conditions and to inhibit anabolic reactions that consume ATP (Hardie 2007). Given that T6P responds strongly to sucrose supply (Lunn et al. 2006), T6P would counter the starvation response mediated by SnRK1 to promote growth and biosynthetic reactions in response to sucrose availability.

The signaling function of T6P and/or AtTPS1 is likely integrated in sugar signaling networks

Increased AtTPS1 levels lead to 3-6 fold higher *ABI4* expression, but on medium with glucose, expression of *ABI4* in *AtTPS1* over-expressors is much reduced compared to wild type (Avonce et al., 2004). Since *abi4* mutants are glucose insensitive (Huijser et al., 2000; Soderman et al., 2000), this can explain the glucose- and ABA- insensitive phenotype of *AtTPS1* over-expressors. In addition, *hxk1,* also glucose insensitive has much increased *AtTPS1* expression on glucose medium. However, it is not clear if this increased *AtTPS1* expression also comes with a reduced level of *ABI4* expression, which if it were the case would explain *hxk1* resistance to glucose (Jang et al., 1997; Jang and Sheen, 1994; Moore et al., 2003). Interestingly *ctr1*, which is also glucose insensitive (Gibson et al., 2001), has increased *TPS1* expression (more than twofold in rosette, log1.3) and much decreased *ABI4* expression (more than eightfold, log3) suggesting that *CTR1* regulates *AtTPS1* gene expression which then leads to reduced *ABI4* expression (Arroyo et al., 2003 and Genevestigator, mutants). To conclude, AtTPS1 may mediate *hxk1* insensitivity to glucose and AtTPS1 appears to act on *ABI4* expression levels.

GENETICS IMPLY TREHALOSE METABOLISM IN PLANT STRESS PROTECTION

Expression of *S. cerevisiae ScTPS1* using either Rubisco or CaMV35S constitutive promoters in tobacco produced drought-tolerant plants (Holmstrom et al., 1996; Karim et al., 2007; Romero et al., 1997). In the case of Holmstrom et al. (1996) and Karim et al. (2007), improved water retention of the leaves underlies this tolerance. Transformation with these constructs, however, led to smaller leaves and leaf area that would also likely improve drought tolerance. Fusion of a variety of TPS and TPP enzymes as well as co-expression of the two enzymes no longer causes the developmental changes typically occurring in plants with high T6P levels, yet the constructs mediate resistance to a variety of abiotic stresses including drought, cold and salt tolerance in both dicots and monocots (Garg et al., 2002; Jang et al., 2003; Karim et al., 2007; Miranda et al., 2007; Figure 6). Because abiotic stress tolerance is mediated by proteins from a variety of sources including *S. cerevisiae* and *E.coli*, the tolerance mechanism must involve the metabolites trehalose and or T6P, or a molecule derived thereof. Trehalose levels in the resistant transgenic lines did not generally correlate with the degree of drought tolerance observed (Garg et al., 2002; Karim et al., 2007). It may be that measurement of whole tissue metabolite content will not reveal localized effects in specific cell types.

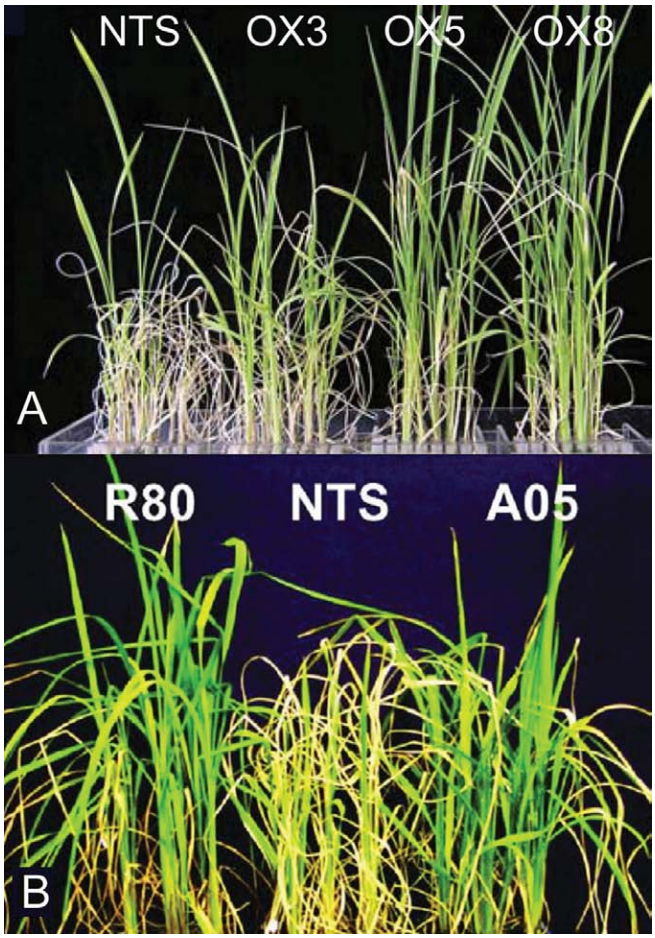

Figure 6. Abiotic stress tolerances mediated by enzymes of trehalose biosynthesis.

A) Transgenic rice plantlets after cold treatment at 6-8°C for 18 days from the OX lines 3,5 and 8 over-expressing the rice TPP *OsTPP1* compared to wild type control NTS. Image reproduced from Ge et al. (2008) with kind permission from Springer Science+Business Media.

B) Transgenic rice plants after 100 h drought stress expressing the TPS/TPP fusion of OtsA and OtsB either targeted to the cytosol and expressed using an abscisic acid promoter (R80) or targeted to the chloroplast and expressed using the *rbcS* promoter (A05) compared to the wild type control (NTS). Image reproduced from Garg et al. (2002) with permission from PNAS.

Targeting of the *S. cerevisiae* ScTPS1 protein to chloroplasts no longer causes developmental aberrations but does still mediate drought tolerance (Karim et al., 2007). T6P accumulation in the cytosol therefore may cause developmental changes typically occurring in plants with high T6P. Alternatively, T6P levels in the chloroplast may be normally low because of low UDPG substrate levels and/or high TPP activity in this compartment - AtTPPA and AtTPPB appear to be targeted to the chloroplasts. Drought tolerance by way of TPS over-expression in chloroplasts suggests that trehalose or T6P accumulation in this compartment is important in triggering drought responses. Tolerance to salt and cold by way of OsTPP1 expression in rice would indicate that trehalose accumulation may be the key to triggering abiotic stress tolerance (Ge et al., 2008).

Expression of the *E. coli OtsA/OtsB* gene fusion yielded rice plants with reduced photo-oxidative damage which maintained photosynthesis during the initial exposure to drought compared to wild type control (Garg et al., 2002). Therefore it can be concluded that certain targets of protection to abiotic stress by trehalose metabolism are found in the chloroplast compartment.

In two transgenic potato lines expressing low levels of *ScTPS1*, drought tolerance correlated with improved water retention during drought (Stiller et al., 2008) as it did in Arabidopsis expressing *ScTPS1* or *ScTPS1* and *ScTPS2* (Karim et al., 2007). In addition in the Arabidopsis overexpressors stomatal conductance was somewhat lower than in the wild type under hydrated conditions, but persisted at the same level under drought conditions whereas in the wild type, stomatal conductance decreased to much lower levels. Leaves of the transgenic potato plants contained fewer stomata than wild type (20-30%) leading the authors of the work to suggest that photosynthesis under initial drought is not limited by stomatal closure in the transgenics as it appears to be in wild type (Karim et al., 2007). This would explain maintenance of photosynthesis under drought of rice plants expressing the *E. coli OtsA/OtsB* fusion (Garg et al., 2002). It further suggests that metabolic changes brought about by trehalose metabolites alter the development of guard cells and possibly also the response of guard cells to drought. In addition, expression of *AtTPS1* was shown to be conspicuously responsive to ABA in guard cells (Leonhardt et al., 2004) indicating a role of trehalose metabolism in guard cell response to the drought-induced ABA pathway.

In conclusion, trehalose metabolites in the chloroplast mediate abiotic stress responses but the processes by which this occurs are not understood. In part, the effect of the metabolites on guard cell development and responses appear involved in responses yielding drought tolerance. This may be linked to cold and salt tolerance, but the mechanistic basis is not yet clear. Knock out mutants of AtTPS5, *tps5*, were found to be thermo-sensitive and AtTPS5 interacts with a conserved transcriptional co-activator MBF1c that is required for thermo-tolerance as well as for a number of other stress responses involving salicylic acid and PR1 and ethylene (Suzuki et al., 2008).

Externally supplied trehalose mediates biotic stress responses. For example spray application of trehalose induces resistance to powdery mildew in wheat (Reignault et al., 2001; Renard-Merlier et al., 2007). Foliar Validamycin A spray also elicits an effective defense response in tomato plants against tomato wilt caused by *Fusarium oxysporum* (Ishikawa et al., 2005). Trehalose does not affect fungal growth directly but instead elicits specific plant responses that effectively inhibit fungal penetration and growth. The plant responses elicited by trehalose may well be different in each plant species. Feeding of trehalose to Arabidopsis similarly induces resistance to *Hyaloperonospora parasitica* pv Wako (Schluepmann et al. 2002 European patent application No. 2002077325.5). Resistance to *H. parasitica* pv Wako is also obtained by expression of *E. coli OtsA* in Arabidopsis. The biotic stress responses mediated by trehalose have not been sufficiently characterized but expression and proteome profiling confirm induction of key components in stress responses (Bae

et al., 2005a; Bae et al., 2005b; Schluepmann et al., 2004). For example expression of *AIG2*, *EDS1*, *EDS5*, *PAD4*, *WAK1*, and *WAK2* was induced (4, 3, 4, 4, 38 and 2.5–fold respectively) in seedlings fed with trehalose compared to sorbitol osmoticum control (Schluepmann et al., 2004 microarray data in the supplement). Authors propose that evolutionary ancient connections in microbes that link nutrient, abiotic and biotic stress perception with re-organization of primary metabolism for quantitative synthesis of trehalose as protectant have been retained in plants (Schluepmann et al., 2004). In extant plants however, trehalose does not accumulate in quantities that would be sufficient for a role as protectant. Instead trehalose and T6P mediate signal transduction, and as such, constitute an important link between nutrient and stress metabolism.

DISSECTION OF CAUSE AND EFFECT

We have previously described the numerous processes in which trehalose metabolism has been implicated. Consequently, because of pleiotropy where T6P levels are modified it has been difficult to separate cause from effect. However, Zhang et al. (2009) observed in seedlings with relatively small increases in T6P (2.5-fold above wild type) and less drastic effects than those caused by gene knockout, clear effects on gene expression consistent with a function of T6P as inhibitor of SnRK1. The impact of T6P on carbon utilization for biosynthetic processes associated with growth and the balance between end-product synthesis and degradation mediated through inhibition of SnRK1 could be the primary cause of the effect of T6P on growth and development. Further dissection of cause and effect over a known time scale using an inducible promoter system would be valuable.

There are likely separate targets of T6P and trehalose. Efforts to separate the two have been masked because trehalose feeding also leads to T6P accumulation sufficient to cause effects on growth and starch accumulation for example. A genetic dissection of the effects of trehalose is therefore an alternative approach. To this effect, mutants in the multiple effects observed when Arabidopsis seedlings are grown on trehalose could be characterized. Several categories of mutants would be expected to emerge from such screens including, for example, loss of growth arrest by trehalose. Such a mutant phenotype could be due to failure to transport trehalose or increased trehalase activity. Alternatively, such mutants could accumulate lower levels of T6P due to an alteration in the processes that control T6P steady states. The most interesting mutants will be those growing on trehalose with low trehalase activity and high endogenous T6P as they could yield further information on downstream effects of T6P. Another potential useful screen on trehalose is selection of mutants that no longer display reversed carbon allocation as this could identify proteins involved in the control of carbon allocation under stress, a topic of research particularly important to agriculture.

A promising approach to studying targets of T6P would be the biochemical dissection of its effect on SnRK1 and AGPase redox activation since these can be reconstituted *in vitro*. In addition, affinity chromatography with either of these molecules as bait has yet to be undertaken and could identify proteins with sequestering activity.

Data suggest that discrete TPS and TPP isoforms may mediate developmental and stress responses possibly through differential T6P or trehalose accumulation. Therefore differential expression of *TPS* and *TPP* genes may result in different effects for differing plant tissues. Clearly, it will be essential to study the biochemical function in addition to expression patterns and subcellular localization of all proteins of trehalose metabolism, including transporters.

TPS and TPP from fungi have been found in large complexes and it is possible that the plant proteins will also operate in protein complexes. Characterization of such complexes would shed light on the regulation of T6P synthesis and its link to development. Further biochemical work will also be necessary to evaluate the hypothesis of metabolite channeling.

CONCLUSION

Trehalose metabolism can be likened to Pandora's box. The two associated metabolites, T6P and trehalose appear particularly crucial in plants but are very hard to monitor. The inhibition of SnRK1 by T6P appears particularly significant in explaining the strong effects of T6P in integrating metabolism with growth and development in growing tissues. It is possible that there are additional targets of T6P, because T6P affects AGPase activation in mature leaves without obvious effects on SnRK1 activity. Activation of starch synthesis via redox activation of AGPase may be the major function of T6P in mature tissue. T6P and trehalose are found in several subcellular compartments but we lack fundamental knowledge concerning their transport. It is proposed that evolutionary old connections known from fungi linking nutrient or other forms of stress, primary metabolism and trehalose synthesis have been retained in plants. Trehalose accumulation in most plants including Arabidopsis is very low however, suggesting that it is no longer a cell protectant. The precursor of trehalose biosynthesis, T6P functions as a sugar signal to inhibit SnRK1 in most tissues except in mature leaves. Conspicuous gene radiation has occurred surrounding T6P metabolism in the genomes of all plants analysed thus far and expression of these genes appears differentially responsive to external cues. Because of its striking effects on carbon allocation and utilization in plants, research into trehalose metabolism has gained much interest recently. In addition, evidence is accumulating on the use of enzymes from trehalose metabolism to engineer crops with increased biomass and grain yield under various forms of stresses. The next five years should see significant advances that will lead to a fuller appreciation of the central importance of trehalose metabolism in plants.

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