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Plant Trehalose-6-Phosphate phosphatase multigene family

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Expansive evolution of the *TREHALOSE-6-PHOSPHATE PHOSPHATASE* gene family in *Arabidopsis thaliana*

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Abstract

Trehalose is a non-reducing sugar used as a reserve carbohydrate and stress protectant in a variety of organisms. While higher plants typically do not accumulate high levels of trehalose, they encode large families of putative trehalose biosynthesis genes. Trehalose biosynthesis in plants involves a two-step reaction in which trehalose-6-phosphate (T6P) is synthesized from UDP-glc and Glc-6-P (catalysed by T6P synthase, TPS), and subsequently dephosphorylated to produce the disaccharide trehalose (catalysed by T6P phosphatase, TPP). In Arabidopsis thaliana, eleven genes encode proteins with both TPS- and TPP-like domains but only one of these (AtTPS1) appears to be an active (TPS) enzyme. In addition, plants contain a large family of smaller proteins with a conserved TPP domain. Here, we present an in-depth analysis of the 10 TPP genes and gene products in Arabidopsis (TPPA-TPPJ). Collinearity analysis revealed that all of these genes originate from whole genome duplication events. Heterologous expression in yeast showed that all encode active TPP enzymes with an essential role for some conserved residues in the catalytic domain. These results suggest that the TPP genes function in the regulation of T6P levels, with T6P emerging as a novel key regulator of growth and development in higher plants. Extensive gene expression analyses using a complete set of promoter-beta-glucuronidase/green fluorescent protein (GUS/GFP) reporter lines further uncovered cell- and tissue-specific expression patterns, conferring spatio-temporal control of trehalose metabolism. Consistently, phenotypic characterization of knock-down and overexpression lines of a single TPP, AtTPPG, points to unique properties of individual TPPs in Arabidopsis, and underlines the intimate connection between trehalose metabolism and abscisic acid (ABA)-signaling.
Introduction

The presence of trehalose in a wide variety of organisms and the existence of different biosynthesis pathways suggest a pivotal and ancient role for trehalose metabolism in nature. The most widely distributed metabolic pathway consists of two consecutive enzymatic reactions, with trehalose-6-phosphate (T6P) synthase (TPS) catalysing the transfer of a glucosyl moiety from uridine diphosphate-glucose (UDP-glc) to glucose-6-phosphate (Glc-6-P) to produce T6P and UDP, and T6P phosphatase (TPP) catalysing dephosphorylation of T6P to trehalose (Cabib and Leloir, 1958; Avonce et al., 2006). Apart from operating as a (reserve) carbon source and structural component in bacteria, fungi, and invertebrates, trehalose also functions as a major stress protectant of proteins and membranes during adverse conditions such as dehydration, high salinity, hypoxia and nutrient starvation (Elbein et al., 2003). Trehalose accumulation is also observed in drought-tolerant non-vascular plants like green algae and liverworts and in a few lower vascular resurrection plants. Until about a decade ago, higher vascular plants were believed to have lost the ability to produce trehalose, but with the emergence of more sensitive assays, genome sequencing, and the use of yeast mutant complementation, minute amounts of trehalose and T6P, and functional plant enzyme orthologs were found (Goddijn et al., 1997; Vogel et al., 1998; Lunn et al., 2006). In addition, heterologous expression and disruption of trehalose metabolism in plants conferred pleiotropic effects, ranging from altered stress tolerance, leaf morphology and developmental timing to embryo-lethality (Holmström et al., 1996; Goddijn et al., 1997; Romero et al., 1997; Eastmond et al., 2002; Schluepmann et al., 2003; Avonce et al., 2004; Satoh-Nagasawa et al., 2006; Miranda et al., 2007; Chary et al., 2008), pointing to an important regulatory function. The intermediate T6P has been highlighted as a novel signal for carbohydrate status (for review see Paul, 2008), positively correlating with sucrose levels, redox-regulated ADP-glucose pyrophosphorylase activity and starch biosynthesis (Lunn et al., 2006). Recently, it was reported that T6P inhibits the activity of the SnRK1 protein kinase to activate energy-consuming biosynthetic processes in growing tissue (Zhang et al., 2009) and that it is required for the onset of leaf senescence (Wingler et al., 2012).

In most bacterial and eukaryotic species, the TPS and TPP activities are found on separate proteins. Recent phylogenetic and biochemical analyses showed that some
archaea and bacteria, such as *Cytophaga hutchinsonii*, express proteins that have both active TPS and TPP domains resulting from gene fusion, suggesting that such prokaryotic bifunctional proteins are the evolutionary ancestors of the large eukaryotic trehalose biosynthesis enzymes in which one or both domains have subsequently lost their catalytic activity (Avonce et al., 2010). The yeast TPP enzyme Tps2, for example, harbours an inactive N-terminal TPS domain and an active C-terminal TPP domain. In contrast to the single *TPS* and *TPP* genes in most micro-organisms, the genomes of higher plants encode a remarkably large family of putative trehalose biosynthesis enzyme homologues. These are commonly classified in three distinct subgroups, according to their similarity to the microbial TPS and TPP proteins and/or presence of specific motifs (e.g. conserved phosphatase boxes) (Thaller et al., 1998; Leyman et al., 2001; Eastmond et al., 2003). Even primitive plants such as the alga *Ostreococcus tauri* and the moss *Physcomitrella patens* already contain members of each of these gene families, pointing to the early establishment and conservation of these proteins in plant evolution (Lunn, 2007; Avonce et al., 2010). In Arabidopsis, the class I TPS proteins (AtTPS1-4) show most similarity to the yeast TPS Tps1, but also have a C-terminal domain with limited similarity to TPPs. However, only one of these, AtTPS1, appears to have heterologous enzymatic TPS activity in yeast (Blazquez et al., 1998; Vandesteene et al., 2010). Strikingly, AtTPS1 is the only class I enzyme with an N-terminal extension that seems to operate as an auto-inhibitory domain (Van Dijck et al., 2002).

The class II TPS proteins (AtTPS5-11) are similar bipartite proteins with a TPS-like domain but a more conserved TPP domain. They appear to lack both heterologous TPS and TPP activity (Ramon et al., 2009). The high level of conservation of putative substrate binding residues in class I and class II proteins, however, suggests that substrates might still bind (Avonce et al., 2006; Lunn, 2007; Ramon et al., 2009; Vandesteene et al., 2010). Together with the specific expression patterns of the class I genes (van Dijken et al., 2004; Geelen et al., 2007; Vandesteene et al., 2010) and the extensive expression regulation of all class II members by plant carbon status (Baena-González et al., 2007; Usadel et al., 2008; Ramon et al., 2009), this suggests tissue-specific regulatory functions for these proteins in metabolic regulation of plant growth and development. Finally, Arabidopsis also harbours a family of 10 smaller proteins (AtTPPA-J; 320-385aa) with limited similarity to the class I and class II proteins (795-942aa). Like class II proteins, they contain the phosphatase box consensus sequences, characteristic of the L-2-haloacid dehalogenase (HAD) super family of enzymes, which
includes a wide range of phosphatases and hydrolases (Thaller et al., 1998). It has been suggested that the origin of these plant TPP genes is different from the origin of the class I and II genes (Avonce et al., 2010) and that plants recruited the TPP genes after their divergence from fungi, most probably from proteobacteria or actinobacteria. Consistently, homologous TPP proteins are present in proteobacteria such as *Rhodobacter ferrireducens* (Avonce et al., 2010). To date, only a few of these single-domain plant TPP proteins have been subject to biochemical characterization, e.g. TPPA and TPPB from Arabidopsis (Vogel et al., 1998), OsTPP1 and OsTPP2 from rice (Pramanik and Imai, 2005; Shima et al., 2007) and RAMOSA3 (RA3) from maize (Satoh-Nagasawa et al., 2006).

The phenotypic alterations observed in plants fed with trehalose or genetically modified in trehalose biosynthesis, suggest a pivotal role for trehalose metabolism in integrating the metabolic status with growth and development. Disruption of the only known active TPS enzyme in Arabidopsis (AtTPS1) results in embryo-lethality (Eastmond et al., 2002) and, when rescued to bridge embryogenesis, causes a strong disruption of vegetative and generative development and ABA-hypersensitivity (van Dijken et al., 2004; Gomez et al., 2010). Overexpressing *AtTPS1* on the other hand renders seedlings sugar- and ABA-insensitive (Avonce et al., 2004; Avonce et al., 2005). These observations strongly link trehalose metabolism with ABA signaling. Interestingly, a mutation of a *TPP* gene in maize, RA3, results in a distinct phenotype, with incorrect axillary meristem identity and determinacy in both male and female inflorescences (Satoh-Nagasawa et al., 2006). Arabidopsis plants with overall increased T6P levels, such as *OtsA* (*E. coli* TPS) overexpression plants, similarly show increased inflorescence branching (Schluepmann et al., 2003; van Dijken et al., 2004).

To better understand why higher plants harbour such a large number of putative TPP proteins, we have made a comprehensive study of the 10 Arabidopsis *TPP* genes and gene products, combining phylogenetic approaches and yeast growth complementation assays, together with a detailed analysis of all 10 *TPP* gene expression profiles in Arabidopsis, and a more detailed single *AtTPP* mutant phenotypic analysis.
Results

Collinearity analysis shows that the *TPP* gene family in eudicots mainly expanded through genome duplication

It has been established that many, if not most plant lineages are paleopolyploids (Masterson, 1994; Cui et al., 2006; Soltis et al., 2009; Van de Peer et al., 2009; Jiao et al., 2011). Current evidence that a genome duplication occurred in the common ancestor of extant seed plants, and another one at the base of the angiosperm lineage (Jiao et al., 2011). In addition, a hexaploidization event (referred to as γ) occurred around the base of the eudicots (Jaillon et al., 2007; Tang et al., 2008). Many eudicot lineages underwent additional genome duplication events, many of them around the Cretaceous-Tertiary boundary, 65 mya (Fawcett et al., 2009; Van de Peer et al., 2009). For instance, two additional genome duplications have occurred within the *Brassicales* lineage, referred to as β and α in Arabidopsis (Jaillon et al., 2007; Tang et al., 2008). Remnants of ancient genome duplications have also been discovered in monocot genomes, e.g. an event shared by *Sorghum* and *Oryza* probably dating back to the base of the Poaceae clade (Paterson et al., 2004), and in the moss *Physcomitrella patens* (Rensing et al., 2008).

Genome duplications have had a profound impact on the expansion of certain gene families in plants, in particular regulatory gene families (Maere et al., 2005; Freeling, 2009; Maere and Van de Peer, 2010). We investigated their impact on the *TPP* gene family by searching for collinearity of genomic segments containing *TPP* genes, using the PLAZA v1.0 platform (Proost et al., 2009). Strikingly, all *TPP* genes in eudicots appear to be linked through collinearity relationships, indicating that the *TPP* gene family in eudicots expanded exclusively through genome duplication. In Arabidopsis, 9 out of 10 *AtTPP* genes, all except *TPPD*, were found in duplicated blocks remaining from the most recent genome duplication (α, *K_s* around 0.7-0.8) (fig. 1A). Moreover, all TPPs were retained in duplicate after the α event, except for *TPPA* and *TPPD*. Whereas *TPPA* still lies in a duplicated block remaining from α, for *TPPD* we could not find back any syntenic relationships dating back to the α event, indicating that the whole duplicated stretch, including the duplicate of *TPPD*, was lost or has
degraded beyond recognition. Deeper collinearity relationships caused by previous genome duplication events were also evident. TPPD, for instance, has a syntenic relationship with TPPB, but this duplicated block is much older ($K_s=1.6$) and originates from an earlier genome duplication (data not shown). Extending our analysis to other species, we found clear collinearity relationships linking all TPPs across the eudicots (see for example fig. 1B) indicating that all eudicot TPP duplicates created after the monocot-dicot split have been derived from genome duplications. Several collinearities among monocot TPP genes were also apparent but their phylogenetic relationship is less clear.

**Collinearity constraints improve the phylogenetic reconstruction of the TPP gene family**

We used the TREEFINDER package (Jobb et al., 2004) to reconstruct a naive phylogeny of the TPP gene family in the green plant lineage (see Material and Methods). The resulting tree is depicted in supplementary fig. S1A. It is in good agreement with the tree calculated with different methods by Avonce et al. (2010). The tree multifurcates in 4 branches, the Physcomitrella TPP genes and three clades of angiosperm TPP genes, all having average to high bootstrap support (78.6 – 98.3) except for the branch containing the Arabidopsis TPPF, TPPG, and TPPA genes which had a much lower bootstrap support value of 52.5.

Next, we sought to impose topological constraints on the tree by taking into account the genome duplication relationships among the TPPs. Based on the collinearity relationships found with PLAZA v1.0 (Proost et al., 2009) and the available knowledge on the timing and phylogenetic position of genome duplications and speciations in angiosperm plants (Fawcett et al., 2009), we were able to severely constrain the topology of the TPP tree (supplementary fig. S1B). Because the phylogenetic interpretation of the collinearities among monocot TPP genes was less clear, we did not use them to constrain the tree topology. The constrained tree was again fed into the TREEFINDER package to resolve the remaining unconstrained branches, using a bootstrap ML analysis with 1000 iterations. The resulting phylogenetic tree of the TPP gene family in the land plants is presented in supplementary fig. S1B. The constrained phylogenetic relationships fit relatively well with the unconstrained phylogeny (see
supplementary fig. S1A). Three major subclades of eudicot TPPs are apparent in both trees, and the within-species phylogenetic relationships among TPP genes are identical. Within the subclades, the between-species phylogenetic relationship of the TPPs is more variable. Papaya (Carica papaya) TPPs are for instance often put outside Arabidopsis-Populus clades in the unconstrained topology, possibly an artifact of slower evolution of these genes in papaya. On two occasions, grapevine (Vitis vinifera) TPPs are similarly put erroneously close together in the unconstrained topology. The fact that all nodes in the unconstrained tree that do not match the constrained tree have relatively low bootstrap support is also an indication that constraining the topology on the basis of collinearity relationships significantly improves the tree.

All 10 putative Arabidopsis TPP enzymes have heterologous TPP activity in yeast

The TPP gene family in higher plants appears to have expanded mainly by whole genome duplication, but it is not clear whether all have maintained enzymatic activity. To assess TPP activity, we have used a Sacharomyces cerevisiae TPP mutant complementation assay. Yeast tps2Δ mutants exhibit thermosensitive growth due to accumulation of high levels of T6P at increased temperature (De Virgilio et al., 1993). Previously, complementation of this thermosensitive growth phenotype was used to isolate the TPPA and TPPB genes from an Arabidopsis cDNA library (Vogel et al., 1998). We have now analysed all putative Arabidopsis TPP enzymes in the yeast tps2Δ mutant strain with efficient expression of the 10 proteins confirmed by western blot analysis. All 10 TPP members were able to complement growth deficiency of the yeast tps2Δ mutant strain at elevated temperature (fig. 2A-C). Based on sequence alignment and the crystal structure of the TPP-related T6P phosphatase protein from the archaeon Thermoplasma acidophilum (TaT6PP) (Rao et al., 2006), a strikingly conserved active site is found in all 10 Arabidopsis TPP proteins (Avonce et al., 2006; Rao et al., 2006; Lunn, 2007). We made an overall representation model with T6P modelled in the active site of Arabidopsis TPPG (fig. 2D). TaT6PP uses the common catalytic reaction mechanism typical for the L-2-haloAcid dehalogenase (HAD) superfamily, with the active site located within the cavity at the interface of two major domains (Rao et al., 2006). Three sequence motifs make up the active site, with the N-terminal DXDX(T/V) motif (motif I, aa7-11 in TaT6PP) being the most conserved. In TaT6PP, a nucleophilic attack of Asp7 (equivalent to D113 in TPPG) on the phosphorus atom of T6P is thought
to result in a phospho-aspartic acid intermediate, followed by hydrolysis of an activated water-molecule, generated by Asp9 (D115 in TPPG). Asp9 might also assist as a general acid catalyst, protonating the oxygen atom of the leaving group (Rao et al., 2006). Here, we provide experimental evidence for the importance of these two conserved aspartate residues for TPP catalytic activity. Mutation of these aspartate residues in TPPG (D113 and D115, TPPG-DYD) to asparagine (similar spatial structure as aspartate but harbouring an uncharged polar R group) (TPPG-NYN) abolished yeast tps2Δ mutant growth complementation (fig. 2A-C; confirmed by bioscreen growth analysis, data not shown).

Spatio-temporal expression profiles suggest functional diversity among the Arabidopsis TPP family

In multicellular organisms, protein function is often tightly linked to tissue- and cell type-specific expression. As all Arabidopsis TPP proteins seem to have T6P-phosphatase activity, it is important to determine their spatio-temporal expression in planta to get an initial clue about their functional specificity. We therefore fused a 2kb promoter sequence of each Arabidopsis TPP gene to a GUS/GFP reporter, and introduced these constructs into Arabidopsis ecotype Columbia (Col-0). For each construct, at least three independent homozygous lines (with single insertion) were selected in the T3 generation, and results of homozygous lines with representative expression patterns are shown. A global overview of the expression patterns of the 10 TPP genes in various organs at different developmental stages was obtained with GUS staining and microscopic analyses (fig. 3, fig. 4 and supplementary fig. S2). TPP gene expression occurred in various and distinct organs, from root tips to leaves. At the seedling level, already soon after germination clear differences in TPP gene expression could be observed, with 6 TPPs expressed in the shoot apical meristem (SAM) and 4 TPPs found expressed in the root apical meristem (RAM) (fig. 3A, fig. 4). At the tissue level, we can see TPPD expression in the root cap, whereas TPPA and TPPG are expressed in the protoderm (fig. 3A). In older plants, some TPPs are more highly expressed in young leaves than in mature ones as seen for TPPA, TPPB and TPPG promoter-GUS lines (fig. 3B, supplementary fig. S2). At the cellular level, some TPPs have particular expression profiles in specific cell types, e.g. TPPG in the stomata and...
trichomes of actively growing young leaves, \textit{TPPH} in the vasculature of all leaves and in stomata, and \textit{TPPJ} in hydathodes (fig. 3B, 3C). During the reproductive stage, 5 \textit{TPPs} are found in flowers, especially in the stamens (fig. 3D, supplementary fig. S2). Specific expression in the anthers is found for \textit{TPPA}, \textit{TPPF}, \textit{TPPG} and \textit{TPPH}, while \textit{TPPJ} is expressed in the filament.

Publicly available expression data (Genevestigator, Zimmerman et al., 2004; Hruz et al., 2008), and eFP browser, (Winter et al., 2007) largely fit with the GUS expression profiles. For example, in accordance with Genevestigator, we detected \textit{TPPA}, \textit{TPPF}, \textit{TPPG} and \textit{TPPH} expression in pollen grains (supplementary fig. S2). Also consistent with Genevestigator expression levels below background, we did not detect \textit{TPPC} and \textit{TPPE} GUS-expression. No GUS staining was observed in developing seeds from any of the reporter lines (data not shown). This broadly agrees with the absence of detectable \textit{TPP} transcripts in developing seeds, except for very low expression of \textit{TPPB}, \textit{TPPG} and \textit{TPPH}, which was restricted to the chalazal endosperm.

Previously, we have performed similar promoter-GUS/GFP analyses for the seven class II TPS proteins (Ramon et al., 2009). To determine whether there is any overlap in the spatio-temporal expression profiles of the different TPS and TPP genes, we have generated a combined expression map where we clustered expression patterns (fig. 4). Whereas the Class II TPS genes have largely overlapping expression profiles, the TPP genes have more specific expression patterns. We can observe some overlap between the expression profiles of specific TPS and TPP genes. \textit{TPS7} and \textit{TPS8} cluster more closely with \textit{TPPA}, \textit{TPPJ} and \textit{TPPG}. They are all broadly expressed with high expression in the leaf primordia, the cotyledons, at the hypocotyl-root junction and in the stamens. From these genes, \textit{TPPA} and \textit{TPS7} are more or less expressed throughout the whole plant. There is also some overlap between the expression profiles of \textit{TPS5}, \textit{TPS6}, \textit{TPS9}, \textit{TPS10} and \textit{TPPB}, \textit{TPPH} and \textit{TPPI}. Also \textit{TPPD} and \textit{TPS11} seem to be co-expressed in the same tissue types, more specifically in the columella cells in the root meristematic zone.

\textbf{Functional analysis of TPPs in Arabidopsis}
To assess the functionality of the TPP proteins in Arabidopsis, we analyzed the phenotypes of Arabidopsis plants disrupted in a single TPP by means of available T-DNA insertion lines. Screening of 23 lines from different T-DNA insertion collections led to the identification of knockout (KO) or knockdown (KD) lines of 8 TPPs, \textit{tppa}, \textit{tppb}, \textit{tppd}, \textit{tppf}, \textit{tppg}, \textit{tpph}, \textit{tppi} and \textit{tppj} (Supplementary fig. S3). Seeds of homozygous insertion lines germinated like wild type Col-0 when sown on soil, and the resulting plants show comparable vegetative and reproductive growth to wild type, with the exception of \textit{tppb} mutants producing a larger rosette (Supplementary fig. S4). Overexpression of the \textit{TPPA}, \textit{TPPF}, \textit{TPPG} or \textit{TPPJ} genes, driven by the CaMV 35S promoter, did not alter the seed germination or vegetative growth characteristics of the plants when grown on soil (fig. S4A). However, overexpression of several of the \textit{TPP} genes did affect floral morphology, leading to a shorter perianth, the floral structure including petals and sepals (as exemplified by \textit{35S::TPPB} plants shown in fig. S4B). The result is that the reproductive organs (pistil and stamen) are not protected or covered by the perianth. Apart from \textit{TPPB}, we also observed this for \textit{TPPC} and \textit{TPPI} overexpression lines (data not shown). As growth phenotypes in the mutant TPP lines were not obvious we investigated the function of the TPP enzymes in the biosynthesis of trehalose by measuring trehalose and T6P contents, along with other sugars and metabolites, in whole seedlings of some of these lines (Table 1).

Remarkably few differences in the levels of T6P, trehalose and other metabolites were observed in the \textit{tpp} mutants compared to wild type. The most notable changes were a lower level of trehalose in the \textit{tppb} plants and the higher sucrose and starch content in \textit{tppi} plants. As the TPPs have cell- and tissue-specific expression \textit{in planta}, it is plausible that the effect of single TPP downregulation on metabolite contents is ‘diluted’ in whole seedling extracts. It is also conceivable that metabolic changes in the cells where \textit{TPP} expression has been down-regulated might have systemic effects that led to compensatory metabolic adjustments in cells that do not normally express the affected \textit{TPP} gene.

\textbf{Mutation of a single TPP in Arabidopsis, \textit{AtTPPG}, causes ABA-related phenotypes}

Early reports have linked ABA-signaling to trehalose metabolism, specifically with the function of TPS1. To investigate whether TPPs are also involved in this
response, we investigated the effects of ABA treatments on germination of \textit{tpp} mutant seeds (fig. 5A). At 7 days after sowing (DAS), some \textit{tpp} mutants responded differentially to increasing concentrations of ABA when compared to the wild type Col-0 control and a 35S::\textit{AtTPS1} line, whose germination is not inhibited by ABA (Avonce et al. 2004). The \textit{tppb} and \textit{tppg} mutants are insensitive to increasing concentrations of ABA, showing even higher germination rates than the 35S::\textit{AtTPS1} positive control line with 20 μM ABA and comparable germination at lower concentrations. The \textit{tppf} mutant, on the other hand, is hypersensitive to ABA.

In order to investigate further the effect of ABA on the \textit{tpp} mutants, we looked at the effect of this hormone in stomatal aperture and closure. In the above-ground organs, TPPG is rather specifically expressed in guard cells, and the \textit{tppg} mutant showed resistance to ABA inhibition of germination. Therefore, we focussed our attention on \textit{tppg} and mutants in its duplicate gene (\textit{tppf}) and a close homologue (\textit{tppa}), with one representative from a distant clade (\textit{tppj}) for comparison (see fig. S1). We also generated and included the overexpressing lines of these TPPs (\textit{TPP-OE}) using a constitutive promoter. Epidermal peels of fully expanded leaves of the above mentioned \textit{tpp} mutants and overexpressing lines were incubated under conditions that promote stomatal opening and then treated with 20 μM ABA to induce stomatal closure. As seen in the germination assay, \textit{tppg} was resistant to ABA, showing no stomatal closure in response to ABA, while the other mutants behaved as the wild type. The OE line responses were similar to wild type with the interesting exception of \textit{TPPF-OE}, which is slightly more sensitive to the ABA treatment (fig. 5B). The decreased sensitivity of seed germination and stomatal opening to ABA in the \textit{tppg} mutant suggests that TPPG might have a particular role in linking trehalose metabolism to ABA signaling in a cell type specific way.

**Discussion**

In Arabidopsis, there appears to be only a single enzyme, TPS1, capable of synthesising T6P, but a great diversity of TPP enzymes able to hydrolyse T6P to trehalose. As many of the TPP proteins show specific spatio-temporal expression patterns, they clearly have great potential to exert control over T6P levels according to
the needs of specific cell and tissue types. Given the substantial and growing evidence that T6P is an essential signal metabolite in plants, with far reaching influence on plant metabolism, growth and development, there is an urgent need to understand the origins and physiological significance of the large number of plant TPP proteins.

Collinearity analysis of the TPP gene family strongly indicates that all eudicot and many monocot TPP genes originate from whole genome duplications. Maere et al. (2005) and Freeling (2009) found that only particular classes of genes preferentially expand through genome duplications in plants, mainly regulatory genes (transcription factors, signal transducers) and genes encoding complex-forming proteins. Similar observations have been made in mammals (Blomme et al., 2006) and other organisms (Maere and Van de Peer, 2010 and references therein). A likely explanation for this phenomenon is offered by the dosage balance hypothesis, which postulates that the stoichiometric quantities of proteins functioning within protein complexes and regulatory/signaling cascades must be preserved in order to ensure proper function (Papp et al., 2003; Birchler et al., 2005; Veitia et al., 2008; Maere and Van de Peer, 2010). This requirement is violated by small-scale gene duplications but fulfilled by whole genome duplications, where all components of such complexes and/or cascades are simultaneously duplicated. Moreover, after genome duplication, balance-sensitive gene duplicates cannot easily be lost, as this would again give rise to dosage balance effects. The particularly strong genome duplication bias in the history of the TPP gene family therefore hints at a potential regulatory role for the TPP proteins.

The next step to further elucidate the functionality of these proteins was to analyze if these 10 TPP genes in Arabidopsis still encode active T6P-phosphatase enzymes. Heterologous expression in yeast showed that all 10 candidate TPP genes encode heterologously active TPP enzymes; apparently all 10 proteins have conserved their enzymatic function during evolution. We also confirmed the essential role of some conserved residues in the catalytic domain as predicted by an archaean crystal structures, by specific site directed mutagenesis.

Analyzing promoter GUS/GFP lines, we found very diverse spatio-temporal expression patterns for the 10 Arabidopsis TPP genes, indicating possible functional diversification. In general, our data correlate well with public available gene expression
datasets, although there were some differences from the spatio-temporal root expression maps published by Brady and coworkers (2007), for example, they did not find detectable levels of expression TPPD in the root cap, or TPPA and TPPG in the epidermis. The apparent discrepancies between our promoter-reporter lines and the transcript profiling data could be explained by differences in GUS/GFP and TPP transcript stability, with some TPP transcripts being rapidly turned over in certain cell or tissue types, raising the possibility that the pattern of TPP gene expression in roots is subject to post-transcriptional regulation.

Since trehalose metabolism and especially the intermediate T6P seem to have profound effects on global plant performance, and interfere with plant growth and development, differential spatio-temporal regulation of the trehalose metabolic genes is plausible. Its connection to sugar sensing/signaling suggests that cellular monitoring of, and responses to, subtle fluctuations of T6P levels are essential for carbon homeostasis and the integration of the nutritional status with plant growth and development. The phylogenetic analysis and the differential expression patterns indicate distinct functionality among the different TPPs; a hypothesis supported by available microarray datasets showing TPP expression regulation by various environmental signals (Kreps et al., 2002; Wang et al., 2003; Contento et al., 2004; Scheible et al., 2004; Thimm et al., 2004; Bläsing et al., 2005; Brenner et al., 2005; Liu et al., 2005; Roth et al., 2006), and by published co-expression analyses that cluster every individual TPP in separate networks associated with genes that identify different functional categories (Ma et al., 2007; Li et al., 2008). Since partial overlap is present in the promoter-GUS expression patterns, some redundancy between the TPP isoforms cannot be excluded. Further research is needed to clarify if the TPPs operate by spatio-temporal regulation of T6P levels and/or have a regulatory function uncoupled from their enzymatic TPP activity. To investigate the later possibility, it will be interesting to investigate whether catalytically inactive forms of the enzyme can complement the phenotypes of the tpp loss-of-function mutants, however, this is outside the scope of the current study. The class II TPS proteins appear to have been present in the green plant lineage from its earliest evolutionary origins and are structurally related to the yeast TPP (Avonce et al., 2006; Lunn, 2007). While the Arabidopsis class II proteins still contain TPP-like domains, suggesting that they might once have had TPP activity, none of these proteins confers heterologous TPP activity (Ramon et al., 2009). We might speculate that the
recruitment of the 10 *TPP* genes encoding active TPP enzymes may have released the class II proteins from their putative function as TPP enzymes, allowing them to evolve new, apparently beneficial, functions, which may or may not be linked to trehalose biosynthesis or T6P signaling. This might explain why there is little similarity in the expression patterns between *TPS* and *TPP* genes.

As is often the case for multigene families, single mutant phenotypes are hard to find, plausibly because of redundancy among family members. Growth phenotypes in normal conditions were not evident for most of the *tpp* mutants, but smaller rosette leaves and later flowering were observed in different *TPP-OE* lines (Supplementary Fig. S4). Metabolic phenotypes were also elusive, at least at a whole-plant or whole-rosette level. Even for abundant metabolites, measuring the levels in specific cell or tissue types, for example by laser capture micro-dissection, is still technically very challenging, and for low-abundance metabolites such as T6P and trehalose is not yet feasible. Emerging technologies such as scanning MALDI-TOF mass spectrometry or FRET-based reporters promise to increase the resolution of metabolite analysis, even down to the single cell level, and might eventually reveal metabolic differences in the specific cell or tissue types where TPP expression has been disrupted.

Interestingly, however, a single TPP knockdown did have a relevant effect when subjected to ABA treatment. The phenotype of Arabidopsis plants deficient in *TPPG*, links its expression in guard cells with a regulatory function in stomatal closure. Although *TPPH* is also expressed in leaf stomata in some growth conditions, the ABA-related stomatal closure phenotype of the *tppg* mutant indicates a unique role for TPPG in this response. Whether the stomatal kinetics of the *tppg* plants are caused by the tissue-specific accumulation of T6P levels in the guard cells (through the loss of the enzyme activity), or by the loss of the TPPG protein itself (which might have a regulatory function, uncoupled from its catalytic activity) will be the subject for further research. Interestingly, former reports with transgenic plants overexpressing yeast *TPS1* also link stomatal regulation during drought stress with trehalose metabolism (Gaff, 1996; Stiller et al., 2008). A co-expression analysis linked *AtTPPG* to *DREB2A* and *DREB2B* expression (involved in drought response) (Li et al., 2008). The *AtTPPG* promoter also contains an ABRE binding site and DRE core motifs (ATHENA, O'Connor et al., 2005); (AGRIS, Palaniswamy et al., 2006).
The opposing effects of ABA on germination of mutant and transgenic plants, with \textit{tppg} mutants being resistant and \textit{TPPG-OE} plants hypersensitive to ABA (Supplementary fig. S5), further corroborate the strong crosstalk present between ABA and trehalose metabolism, previously reported by Avonce et al (2004) and Gomez et al (2010). Surprisingly, the duplicated homolog of TPPG, TPPF, appears to display an opposite activity during the germination process, suggesting that control of T6P levels might not be its unique function, but that the enzyme functionally diverged. Another possibility is that TPPF and TPPG may differ in their $K_m$ for T6P or other kinetic and regulatory properties, and thus have different effects on T6P and/or trehalose levels when up- or down-regulated independently. ABA is known to inhibit the induction of cell wall hydrolases that weaken and break the micropylar endosperm, allowing the radicle to emerge from the seed during the germination process (Müller et al., 2006; Gimeno-Gilles et al., 2009). Consistent with the observed phenotype, \textit{TPPG} expression is localized in the micropylar endosperm of the developing seed (http://www.seedgenenetwork.net). The differences in ABA sensitivity of the \textit{tppg} and \textit{TPPG-OE} mutants also fit with former observations of \textit{TPS1} Arabidopsis mutant lines, with contrasting ABA sensitivity correlating with the differences in T6P content. Three TILLING alleles of \textit{tps1} with reduced T6P levels confer hypersensitivity to ABA in germination assays and defects in ABA-regulation of stomatal pore aperture regulation (Gomez et al., 2010), whereas \textit{AtTPS1} overexpressing Arabidopsis seedlings exhibit ABA-insensitivity in germination assays and abolished glucose-induced ABA accumulation on 6% glucose (Avonce et al., 2004; Avonce et al., 2005). These observations all indicate that there is a strong link between T6P levels and sensitivity to ABA.

In conclusion, our analyses point to the functional divergence of the large multigene family of \textit{TPPs} in the model plant \textit{A. thaliana}. All 10 genes originate from whole genome duplications, rather than from small-scale gene duplications, suggesting that this family may exhibit important regulatory functions. Detailed gene expression profiles indicate that tissue-specific regulation of T6P levels might be the key function of this class of 10 catalytically active proteins. The ABA-related stomatal phenotype of a single \textit{TPP} mutant in Arabidopsis, finally, illustrates the functional divergence and specificity of a single TPP protein.
Materials and Methods

Phylogenetic and collinearity analyses

The genes used for the collinearity analysis and for the phylogenetic reconstruction of the TPP gene family in the green plant lineage were collected using the PLAZA v1.0 platform (Proost et al., 2009). Sequences were available for the following land plants: *Arabidopsis thaliana*, *Carica papaya*, *Populus trichocarpa*, *Vitis vinifera*, *Oryza sativa*, *Sorghum bicolor*, and the moss *Physcomitrella patens*. The sequence of an *Ostreococcus lucimarinus* TPP gene belonging to the Chlorophyta was added as an outgroup. Amino-acid level multiple sequence alignment was performed with MUSCLE (Edgar, 2004). For phylogenetic reconstruction, we used the TREEFINDER program (Jobb et al., 2004), a program that can infer maximum likelihood phylogenies based under a variety of models of sequence evolution with the possibility to specify topological constraints and perform tree calibration. We used the JTT protein substitution model (Jones et al., 1992) in combination with the discrete gamma heterogeneity model (Yang, 1994) with 5 rate categories, as suggested by the TREEFINDER Model Proposer. Bootstrap analyses were performed with 1000 maximum likelihood iterations.

Modeling of T6P in the active site of AtTPPG

We modelled binding of the ligand trehalose-6-phosphate (T6P) to the active site of Arabidopsis TPPG. A model of TPPG was constructed with MODELLER 9v8 (http://salilab.org/modeller/) using the crystal structure of the TPP-related protein from the archaean *Thermoplasma acidophilum* (TaT6PP, PDB ID: 1U02), a Mg$^{2+}$-dependent phosphatase belonging to the L-2-haloacid dehalogenase (HAD) superfamily (Rao et al., 2006) as a template. T6P (taken from PDB ID: 1BYK) (Hars et al., 1998) was manually placed into the active site of TPPG with COOT (Emsley et al., 2010) according to Rao et al. (2006) and afterwards energy-minimized using CNS (Brünger et al., 1998).

Yeast growth complementation assay

For the yeast growth complementation assay, the yeast (*Saccharomyces cerevisiae*) W303-1A wild-type strain (*Matα* leu2-3, 112 ura3-1 trpl-1 his3-11,15 ade2-1 can1-100 GAL SUC2), the *tps1Δ* deletion strain YSH290 (W303-1A,
tps1Δ::TRP1) and the tps2Δ deletion strain YSH448 (W303-1A, tps2Δ::HIS3) (Hohmann et al., 1993) were used (see also (Ramon et al., 2009; Vandesteene et al., 2010). The different TPP CDS (confirmed by, and if necessary corrected to, the sequence alignment to the TAIR Arabidopsis Genome Initiative Coding Sequence dataset), were amplified from A. thaliana cDNA and cloned in a modified yeast multicopy pYX212 plasmid with an HXT7 promoter and URA3 marker, without stop codon and in frame with C-terminal double HA tag (primers, see supplementary table S1). Primers used for AtTPPG-NYN mutagenesis were 5’ATAGCTGTGTTTCTAAATTATAATGGAACACTTTCTC3’ and 5’GAGAAAGTGTTCCATTATAATTTAGAAACACAGCTAT3’. As a control for the complementation assay, the tps2Δ strain was also transformed with the empty vector and the yeast TPP TPS2 (Vogel et al., 1998; Zentella et al., 1999; Ramon et al., 2009). Yeast transformation was performed using the one-step method as described by Chen et al. (1992). Cultures of the transformed tps2Δ and WT control strains were grown overnight at 28°C on SD-ura containing 2% glucose and drop-assays were performed on SD-ura containing 2% glucose and galactose. Transformants were spotted at an OD600 of 1 and incubated at 28°C (control) or 38.6°C and analyzed after two days. For growth curves, pre-cultures were grown in SD-ura with 2% glucose at 28°C. Samples were then diluted to OD600 0.100 in 200μl SD-ura in 100-well Honeycomb2 plates (Thermo Electron Corporation) in three replicates. Blanks and appropriate growth controls were also included. The transformed tps2Δ strains and wild type strain were grown in SD-ura with 2% glucose at 28°C (control) and 38.6°C. Growth was followed by OD measurements every 15 min with continuous shaking in the Automated Microbiology Growth analysis System (Bioscreen C) (Oy Growth Curves AB Ltd).

Western blot analysis of heterologous protein expression

After overnight growth, 50ml yeast cell cultures were harvested and resuspended in ice-cold lysis buffer (500 μl 1xPBS, 0.1% Triton X-100, 10% glycerol, 2.5 mM MgCl2, 1 mM EDTA, pH8) containing protease inhibitors (Complete EDTA-free, Roche). Cells were lysed by vortexing (0.5 mm glass beads). After centrifugation at 4°C for 10 min at maximum speed, protein concentrations in the supernatant were determined using the dye-binding assay (Bradford, 1976) and the soluble extract was subjected to immunoprecipitation using anti-HA rat antibodies 3F10 (Roche) and
protein G-agarose beads (Roche). Bead-bound proteins were collected by centrifugation at 4°C (30s, 2000 rpm), and washed several times with lysis buffer. Amounts of immuno-precipitated protein did not vary more than 3-fold between samples. SDS-PAGE sample buffer was added, followed by boiling for 5 min. After centrifugation, equal amounts of proteins were loaded on a Mini-Protean precast gel (Bio-rad) together with the protein standard (Invitrogen, SeeBlue Pre-stained protein standard) in a Tris/glycine/SDS running buffer and analyzed using immunoblotting. For detection of HA-tagged proteins, we used HRP-coupled anti-HA High Affinity rat antibodies (1/1000; Roche). Protein bands close to the predicted size of the respective TPP-HA fusion protein were considered to be full length.

**Promoter GUS/GFP constructs**

To study the expression patterns of all TPPs in vivo, we amplified and fused the promoter/5’UTR sequences of around 2kb of all TPPs to GUS/GFP reporter genes in the pHGWFS7 vector (Karimi et al., 2002) using the GatewayTM technology (Invitrogen) according to manufacturer’s instructions. Vectors were obtained from the Plant Systems Biology department (VIB, UGhent) (http://www.psb.ugent.be/gateway). Primers used are listed in supplementary table S1. Wild type *A. thaliana* accession Columbia-0 plants were transformed by floral dip with *Agrobacterium tumefaciens* (C58C1) containing the promoter constructs (Clough and Bent, 1998). Homozygous plants for at least three (independent and single-insertion) transformed Arabidopsis lines were selected for each TPP promoter construct. Seeds were surface sterilized using chlorine and sown in petri dishes on full strength Murashige and Skoog Salt Mixture including vitamins (MS) medium (4.4g/l) with MES (0.5g/l) and solidified with Phytagar (8g/l) (Duchefa) with HygromycinB following a modified protocol from Harrison et al. (2006).

**Histochemical and histological analysis**

For detailed analysis of seedlings and roots, seedlings were grown for 2 days after germination on vertical plates containing 0.5xMS and 1% (w/v) Suc in, continuous light. For shoot analysis, plants were grown for 15 days after germination, on horizontal plates (as above) with a 16h-8h day/night cycle. For flower analysis, plants were grown on soil for 5-6 weeks with a 16h-8h day/night cycle. The beta-glucuronidase (GUS)-staining assays were performed according to Beeckman and Engler (1994). Seedlings,
shoots and flowers were afterwards cleared by mounting them in 90% lactic acid (Acros Organics) on glass microscope slides. Samples were analyzed by differential interference contrast microscopy (Olympus BX51). Qualitative GUS expression levels of the 10 TPP genes in different tissues, were clustered by the Genesis program (Sturm et al., 2002).

Characterization of AtTPP mutants and AtTPP-OE lines

T-DNA insertion lines were obtained from NASC (Alonso et al., 2003) and genotyped for homozygosity with specific primers according to T-DNA express: Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) (supplementary Table S2). AtTPP-OE lines were generated by cloning the corresponding TPP CDS with a 2xHA-tagged sequence from the pYX212 vectors generated for the yeast growth complementation assay (primers, supplementary table S2), into a pDONR221 donor vector, followed by subcloning in a pK7WG2 expression vector with 35S promoter (Karimi et al., 2007) using the Gateway™ technology (Invitrogen) according to manufacturer’s instructions. Arabidopsis accession Col-0 plants were transformed with the expression vector and selected on kanamycin. Homozygous, independent-single insertion lines were selected by screening of T3 progeny.

TPP expression in the mutant and OE lines was tested with real time qRT-PCR. RNA was extracted with an RNeasy kit (Qiagen), and cDNA prepared from 1 µg of total RNA using an iScript cDNA synthesis kit (Bio-Rad) before analysis on a LightCycler 480 Real-Time PCR instrument (Roche Applied Science) using SYBR Green I Master reagent (Roche Applied Science) according to manufacturer’s instructions. All reactions were done in triplicate. Relative expression levels of target genes were quantified with specific primer sets (supplementary table S2), and normalized to cDKA 1;1 (At3g48750) and EEF1α4 (At5g60390) expression levels.

Metabolite measurements

Between 10-15 plants grown for 11 DAG in vertical plates on standard half strength MS supplemented with 1% sucrose under continuous light (110 µE.m⁻².s⁻¹ photosynthetically active radiation) at 22°C, were pooled and frozen immediately in liquid nitrogen. Tre6P, phosphorylated intermediates and organic acids were measured
by liquid chromatography coupled to tandem mass spectrometry as described in Lunn et al. (2006). Sucrose, glucose and fructose contents were measured in ethanol extracts as described by Jelitto et al. (1992) and starch was measured in the ethanol insoluble residue according to Hendriks et al. (2003) Trehalose was measured in the same plant extracts used for Tre6P measurements (Lunn et al., 2006) by fluorimetric assay with coupling via trehalase and glucose oxidase and Amplex Red (Invitrogen).

ABA germination assay

AtTPP, p35S::TPS1 and Col-0 wild type seeds were sown in 0.5x MS supplemented with 1% sucrose and different concentrations (0.0, 2.5, 10.0 and 20.0 µM) of ABA (Sigma). The % of germinated seeds was scored daily for 7 days.

Measurements of stomatal aperture

The first pair of fully expanded leaves of 3 weeks old plants, grown in soil 16h-8h day/night cycle, was detached and incubated in a stomata opening buffer (10mM MES-KOH pH6.15, 10mM KCl) for 2 hours under continuous light. 20µM of ABA (0µM in controls) was added to the buffer and leaves were incubated 2 hours more. Peels of the abaxial side of the leaf were taken by pasting the abaxial side of the leaf to a microscope slide with double-sided sticky tape, the mesophyll cells were removed by gently scraping the leaf with a blade and the abaxial epidermis could be visualized in a microscope (Olympus BX51). Pictures of stomata were taken and pore aperture was measured using ImageJ software.

Supplemental material

Supplementary Figure S1. Phylogenetic tree of plant TPPs. A. Phylogenetic tree of land plant TPP genes without any imposed topological constraints. Inner values denote bootstrap support. The branch length scale is denoted underneath the figure. B, Phylogenetic tree of land plant TPP genes with topological constraints. Inner values denote bootstrap support. The branch length scale is denoted underneath the tree. Stars represent topological constraints and the 10 members of the TPP gene family of Arabidopsis are highlighted.
Supplementary Figure S2. Expression of TPPs in pollen grains. Only TPP promoter-GUS lines with detectable GUS-expression in pollen grains are shown.

Supplementary Figure S3. qPCR on TPP mutant and overexpressing lines. A, Relative expression of the corresponding TPP in mutants with T-DNA insertions. B, Relative expression of the corresponding TPP in generated overexpression lines.

Supplementary Figure S4. Shoot Phenotype of TPP mutant lines. A, Shoot of 12dag TPP-KO plants grown in a growth chamber with a 20h/4h day/night regime. B, Shoot of 12 dag TPP-OE plants grown in the same conditions as A. C, Example of flowers of a 32dag TPP-OE line grown in the greenhouse under a 18h/6h day/night regime.

Supplementary Figure S5. Germination inhibition assay with ABA in different concentrations shows hypersensitivity to ABA in TPPG-OE.

Supplementary Table S1. Primers used for cloning of promoter and CDS.

Supplementary Table S2. T-DNA lines; primers to detect T-DNA insertions; primers to check TPP expression levels in mutant lines of the 10 *AtTPP* genes.

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Figure legends

**Figure 1. Collinearity of the Arabidopsis TPP genes.** A. Collinearity information extracted from the PLAZA v1.0 platform. For each multiplicon, the PLAZA v1.0 multiplicon ID and corresponding $K_S$-value are depicted. The $K_S$-values for all multiplicons shown here are typical of duplicated blocks remaining from the most recent genome duplication in Arabidopsis (Fawcett et al., 2009). B. Example of collinearity relationships among TPPs across the eudicots in multiplicon 894, identified from the PLAZA v1.0 database using $A/TPPC$ as the seed gene in the Skyline Plot utility. Genes with the same colour represent members of the same gene family that are present in several of the homologous blocks (also known as anchor points). TPP genes are coloured black. The ratio of collinear regions expected for Vitis:Poplar:Carica:Arabidopsis on the basis of their genome duplication history is 1:2:1:4 (Fawcett et al., 2009), very close to the observed ratio of 1:2:1:3, indicating that very little loss has occurred in this clade. Only the collinear region of $TPPD$ created by the most recent whole genome duplication in Arabidopsis appears to have been lost.

**Figure 2. The 10 Arabidopsis TPP candidates display heterologous TPP activity.** A. Yeast $tps2\Delta$ growth complementation assay of TPP Arabidopsis proteins at 38.6°C with strains grown at a permissive temperature of 28°C as control treatment. $tps2\Delta$ yeast strains expressing the $TPPG-NYN$ mutant (Asp113 and Asp115 of wild type TPPG were mutated to Asn) showed heat sensitive growth phenotype, indicating the double mutation causes loss of TPPG catalytic activity. B. For the spot assay, cells of all samples were diluted to OD$_{600}$ 1 and incubated at 28°C and 38.6°C. C. Expression of the HA-tagged proteins was confirmed by Western blot analysis. Asterisks indicate the full-length tagged protein, based on the predicted mass (TAIR) and the pre-stained protein standard. D. Overall representation model of the TPPG protein (green), and detail of T6P (blue) modeled in the active site of TPPG. Catalytic important residues near the active site are reported in green ball and stick figures. The two Asp of Motif I, which are predicted to play a crucial role in the substrate binding and the catalytic mechanism, are indicated in yellow. Asp113 and Asp115 of TPPG (TPPG wild type) were mutated to Asn (TPPG-NYN). Figures prepared with PYMOL (DeLano, 2002).
Figure 3. Differential expression profiles of Arabidopsis TPP genes. A. Overview of TPP expression patterns in young seedlings, with images of whole seedlings, and detailed views of the shoot meristemic zones and the root tips. Specific expression profiles of each TPP in seedlings can be noticed. B. Expression patterns of the TPP genes in shoots. Some TPP genes are expressed in the shoot apical meristem (SAM) as well as in the leaves, e.g. TPPA and TPPG have an epidermal pattern, TPPH is present in vasculature, TPPJ in hydathodes. C. Detailed TPPG expression profile in leaves. TPPG is expressed in trichomes and stomata. D. Overview of TPP gene expression in flowers. Only TPP promoter-GUS lines with detectable GUS-expression in flowers are shown.

Figure 4. Clustering of the qualitative expression levels of the trehalose metabolism genes in several tissues of Arabidopsis thaliana. TPS7-8 cluster more closely with TPPA-J-G, TPS5-6-9-10 with TPPB-H-I and TPS11 with TPPD, reflecting similarities in tissue specificity. Clustering was performed with the Genesis program (Stum et al., 2002). Light red= strong expression, dark red= weak expression, black= no expression.

Figure 5. Response of TPP Arabidopsis mutants to ABA. A. Germination inhibition assay with ABA. Seeds of available TPP mutants, TPSI-OE and Col-0 wild type plants were sown in different ABA concentrations; the percentage of seed germination was calculated 7 days after sowing (das). tppb and tppg seedlings were able to germinate at high levels of ABA, showing resistance to the inhibition of germination by ABA as seen with the positive control line TPSI-OE. The opposite is observed for the tppf line. B. Stomatal aperture size in epidermal strips isolated from some tpp mutants and overexpressing plants in the absence or presence of 20μM of ABA. Among all the TPP mutants tested only tppg stomata show resistance to ABA promotion of closure.
### Table 1. Levels of T6P, sugar phosphates, sugars, trehalose and starch in TPP mutants.

Measurements were done in 12 DAG plants grown on vertical plates supplemented with 1% sucrose under continuous light. n=4 independent experiments, ±SD. *Data of shoot only.

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<th>tppg</th>
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<th>tppb*</th>
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Figure 1. Collinearity of the Arabidopsis TPP genes. A, Information was extracted from the PLAZA v1.0 platform. For each multiplex, the PLAZA v1.0 multiplex ID and corresponding Ks-value are depicted. The Ks-values for all multiplexes shown here are typical of duplicated blocks remaining from the most recent genome duplication in Arabidopsis (Fawcett, 2009). B, Example of collinearity relationships among TPPs across the eudicots. The eudicot part of multiplex 894 was found after a search with AtTPPC in the Skyline Plot utility of the PLAZA v1.0 platform. Genes with the same colour represent members of the same gene family that are present in several of the homologous blocks (also known as anchor points). TPP genes are coloured black. The ratio of collinear regions expected for Vitis:Poplar:Carica:Arabidopsis on the basis of their genome duplication history is 1:2:1:4 (Fawcett, 2009), very close to the observed ratio of 1:2:1:3, indicating that very little loss has occurred in this clade. Only the collinear region of TPPD created by the most recent whole genome duplication in Arabidopsis appears to have been lost.
Figure 2. The 10 Arabidopsis TPP candidates display heterologous TPP activity. A, Yeast tps2Δ growth complementation assay of TPP Arabidopsis proteins at 38.6°C with strains grown at a permissive temperature of 28°C as control treatment. tps2Δ yeast strains expressing the TPPG-NYN mutant (Asp113 and Asp115 of wild type TPPG were mutated to Asn) showed heat sensitive growth phenotype, indicating the double mutation causes loss of TPPG catalytic activity. B, For the spot assay, cells of all samples were diluted to OD600 1 and incubated at 28°C and 38.6°C. C, Expression of the HA-tagged proteins was confirmed by Western blot analysis. Asterisks indicate the full-length tagged protein, based on the predicted mass (TAIR) and the pre-stained protein standard. D, Overall representation model of the TPPG protein (green), and detail of T6P (blue) modeled in the active site of TPPG. Catalytic important residues near the active site are reported in green ball and stick figures. The two Asp of Motif I, which are predicted to play a crucial role in the substrate binding and the catalytic mechanism, are indicated in yellow. Asp113 and Asp115 of TPPG (TPPG wild type) were mutated to Asn (TPPG-NYN). Figures were prepared with PYMOL (DeLano, 2002).
Figure 3. Differential expression profiles of Arabidopsis TPP genes. A, Overview of TPP expression patterns in young seedlings, with images of whole seedlings, and detailed views of the shoot meristemic zones and the root tips. Specific expression profiles of each TPP in seedlings can be noticed. B, Expression patterns of the TPP genes in shoots. Some TPP genes are expressed in the shoot apical meristem (SAM) as well as in the leaves, e.g. TPPA and TPPG have an epidermal pattern, TPPH is present in vasculature, TPPJ in hydathodes. C, Detailed TPPG expression profile in leaves. TPHD is expressed in trichomes and stomata. D, Overview of TPP gene expression in flowers. Only TPP promoter-GUS lines with detectable GUS-expression in flowers are shown.
Figure 4. Clustering of the qualitative expression levels of the trehalose metabolism genes in several tissues of Arabidopsis thaliana. TPS7-8 cluster more closely with TPPA-J-G, TPS5-6-9-10 with TPPB-H-I and TPS11 with TPPD, reflecting similarities in tissue specificity. Clustering was performed with the Genesis program (Sturm, 2002).
Figure 5. Response of TPP Arabidopsis mutants to ABA. A, Germination inhibition assay with ABA. Seeds of available TPP mutants, TPS1-OE and Col-0 wild type plants were sown in different ABA concentrations; the percentage of seed germination was calculated 7 days after sowing (das). tppb and tppg seedlings were able to germinate at high levels of ABA, showing resistance to the inhibition of germination by ABA as seen with the positive control line TPS1-OE. The opposite is observed for the tppf line. B, Stomatal aperture size in epidermal strips isolated from some tpp mutants and overexpressing plants in the absence of presence of 20μM of ABA. Among all the TPP mutants tested only tppg stomata show resistance to ABA promotion of closure. *p<0.05, **p<0.01.