Redundant and non-redundant roles of the trehalose-6-phosphate phosphatases and energy-responses in Arabidopsis

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Keywords: Trehalose-6-phosphate phosphatase, multigene family, TPPA, TPPB, TPPG, sugar signaling, leaf area, cell division and atrichoblast

The Arabidopsis trehalose-6-phosphate phosphatase (TPP) gene family arose mainly from whole genome duplication events and consists of 10 genes (TPPA-J). All the members encode active TPP enzymes, possibly regulating the levels of trehalose-6-phosphate, an established signaling metabolite in plants. GUS activity studies revealed tissue-, cell- and stage-specific expression patterns for the different members of the TPP gene family. Here we list additional examples of the remarkable features of the TPP gene family. TPP-J expression levels seem, in most of the cases, differently regulated in response to light, darkness and externally supplied sucrose. Disruption of the TPPB gene leads to Arabidopsis plants with larger leaves, which is the result of an increased cell number in the leaves. Arabidopsis TPPA and TPPG are preferentially expressed in atrichoblast cells. TPPA and TPPG might fulfill redundant roles during the differentiation process of root epidermal cells, since the tppa tpgg double mutant displays a hairy root phenotype, while the respective single knockouts have a distribution of trichoblast and atrichoblast cells similar to the wild type. These new data portray redundant and non-redundant functions of the TPP proteins in regulatory pathways of Arabidopsis.

Trehalose is a common disaccharide in bacteria, fungi and invertebrates, where it serves as a carbon source, structural component and stress protectant.1 The most widespread trehalose biosynthesis pathway consists of two enzymatic reactions mediated by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP).2 Although the amounts of trehalose and its intermediate trehalose-6-phosphate (T6P) are very scarce in higher vascular plants, plant genomes do contain multiple TPS and TPP genes. In Arabidopsis thaliana, trehalose biosynthesis genes are classified in three subfamilies based on their sequence homology to microbial TPS and TPP genes.3,6 Class I genes (AtTPS1-4) display the highest similarity to the yeast TPS, with AtTPSI encoding the only active TPS enzyme.7,8 Class II genes (AtTPS5-AtTPS11) are more similar to the yeast TPP, but do not seem to encode any active TPS or TPP enzyme.9-11 The TPP family members (AtTPPA-J) have the conserved phosphatase boxes, typical for TPP enzymes.12 The ancestor of the TPP family has probably been acquired by horizontal gene transfer from certain archaea or bacteria13 and recently, its members were shown to encode functional TPP enzymes in Arabidopsis.14

Introducing heterologous trehalose biosynthesis genes in plants leads to increased stress tolerance and altered growth and morphology, while knocking out trehalose biosynthesis genes results in embryo-lethality and irregular branching of inflorescences.3,14-18 Opposite phenotypes were obtained when either TPS or TPP enzymes were introduced in plants, pointing to an important role for T6P, the intermediate molecule in the biosynthesis pathway. It is now clear that T6P is an important signaling molecule, regulating the carbon status and starch biosynthesis in plants.3,19,20

We showed in a recent study with the aid of a collinearity analysis that the Arabidopsis TPP gene family mainly originated from whole genome duplications. TPP activity was assayed for the 10 TPP proteins using a complementation assay in yeast. All the TPP members can be considered active TPP enzymes since they restore growth of the yeast tps2Δ strain at elevated temperature. Promoter-GUS studies revealed tissue-, cell- and stage-specific

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Submitted: 11/01/12; Revised: 12/10/12; Accepted: 12/11/12
http://dx.doi.org/10.4161/psb.23209
Citation: Van Houtte H, López-Galvis L, Vandesteene L, Beeckman T, Van Dijck P. Redundant and non-redundant roles of the trehalose-6-phosphate phosphatases in leaf growth, root hair specification and energy-responses in Arabidopsis. Plant Signal Behav 2013; 8: e23209.
expression patterns for each of the TPP genes, indicating that TPP proteins may fulfill important regulatory functions by locally controlling T6P levels. Moreover, the functional diversity of the TPP family in Arabidopsis was demonstrated by the altered ABA-sensitivity of the tppg mutant.14

Plants use the metabolite T6P to signal their sugar status and to regulate their carbon use.5,20,21 As such, T6P levels in the different plant organs, tissues and cell types, and upon environmental changes have to be tightly controlled. Here, we investigated whether a varying sugar supply and/or light program affect TPP gene expression in Arabidopsis seedlings. Therefore, promoter TPP::GUS-GFP lines were grown for 7 d on standard MS culture plates (described in ref. 14), supplemented with 0% and 3% sucrose and subsequently kept in the dark or continuous light for 3 d. GUS stained seedlings showed that starvation conditions (darkness and 0% sugar) led to a downregulation of TPPA, TPPD, TPPG, TPPH and TPII expression in the root tip (Fig. 1). The absence of light seemed to have a bigger impact on TPPA and TPII expression than a lack in sucrose (Fig. 1), while the opposite was noticed for TPPD expression (Fig. 1). Interestingly, the root expression pattern of TPPA in the absence/presence of light and sugars is highly similar to the one of TPPG, whereas the remaining, above-mentioned TPPs display distinct root expression profiles. TPPC, TPPE and TPPF genes are not expressed in roots.14 The variable GUS staining patterns observed in the root tips of promoter TPP::GUS-GFP lines suggest a subtle, mostly unique regulation of TPP gene expression in response to altered sugar availability and light conditions. These findings are indications that the 10 TPP enzymes in Arabidopsis could function in local networks, integrating environmental signals with metabolic processes, through the breakdown of T6P.

TPPs are known to influence plant growth and development. We found that altering the gene expression of one of the plant endogenous TPPs, TPPB, affects the shoot size of Arabidopsis plants. Disrupting the TPPB gene leads to a significant increase in leaf area, as seen in the tppb-1 knockout (SALK_037324,14,22) and the tppb-2 knockdown (Sail_191F08,14,23) after a growth period of 21 d on MS culture plates (Fig. 2A and B). TPPB-1 and TPPB-2 overexpressing Arabidopsis plants14 showed the opposite phenotype in vitro (Fig. 2A and B). In addition, tppb mutants display on average 2 more leaves than Wt plants, whereas TPPB overexpressors seem delayed in growth (Fig. 2B). TPPB is expressed in the shoot apical meristem and at the basal end of young, expanding leaves.14 This expression profile resembles to the one in developing leaves of pCYCB1;1-D-box:GUS lines used for tracking the cell proliferation zone, which gradually disappears from the leaf tip on.14 These expression profiles suggest that TPPB rather interferes with leaf growth during early leaf development than during the maturation stages. Since the initial growth phase in leaves is mostly driven by cell divisions,25 we investigated whether the tppb leaf phenotype was due to an increased cell number or due to a larger epidermal cell area. The first pair of true leaves from 21-d-old mutants, grown on MS culture plates, were prepared for microscopic analysis as described in De Veylder et al.26 Images were taken from basal and apical sections of the abaxial epidermis and analyzed by imageJ. Leaves of the tppb-1 and tppb-2 mutants

**Figure 1.** Histochemical localization of GUS activity in root tips of promoter TPP::GUS-GFP lines under different sugar and light conditions. 7-d-old seedlings were grown on MS media supplemented with 0% and 3% sucrose (SUC) and kept for three additional days in continuous light or dark before sampling.
were 17 to 30% larger than the ones from the Wt (Table 1). The estimated cell number per leaf was significantly higher in the *tppb* mutants, displaying 13% to 37% more cells than the Wt, while the epidermal cell size seemed unaffected (Table 1). Since the majority of pavement cells originate from asymmetric divisions of the stomatal lineage, stomatal indexes were determined as well, but no significant differences were observed (Table 1). In contrast with the *tppb* knockouts, the leaf area of *TPPB-1* plants was reduced by 22% compared with the Wt, while the leaf area of the *TPPB-2* line seemed indistinguishable from Wt Col-0 in this assay (Table 1). The reduced leaf size of the *TPPB-1* overexpressor seemed attributed to a decrease of 13% in the estimated cell number (Table 1). These data indicate that the increased shoot size of the *tppb* mutants could be the result of higher cell division rates or due to a delay in the initiation of cell differentiation. In opposite, a lower cell division frequency or an earlier onset of cell expansion might have caused the presence of smaller leaves in the *TPPB-1* overexpressing line. A possible role for the trehalose metabolism in the cell cycle regulation of *Arabidopsis* plants has been suggested earlier. TPS1, the only enzyme that catalyzes the synthesis of T6P, is known to interact with the cell cycle-dependent kinase CKA1;1, encoding a kinase which associates with the spindle and cytoskeletal structures necessary for cytokinesis. Moreover, embryos of the *tps1* mutant show a decreased cell division rate, which could be the result of impaired trehalose signaling.33 Alternatively, the trehalose metabolism might, as an integrator of the nutritional status and growth, influence the regulation of cell division.34 The exact role(s) of *TPPB* and the trehalose metabolism in the cell cycle regulation still remains elusive.

Another example that demonstrates the importance of TPP proteins during plant development, is the hairy root phenotype of the *tppa tppg* double mutant. The root epidermis of *Arabidopsis* Wt plants contains files with trichoblasts (root hair-producing cells) and files with atrichoblasts (hairless cells). These two types of files are located at distinct positions within the root, which implies that the fate of root epidermal cells depends on the location rather than the lineage.35 When analyzing roots of the *promoter TPP::GUS-GFP* lines (described in ref. 14), *TPPA* and *TPPG* expression was seen in the atrichoblast cells of the root elongation and meristematic zones, respectively (Fig. 3A). These expression patterns suggest that local *TPP* transcription might be important for proper development of atrichoblast cells. When looking at the single *tppa* (GABI_016E11,14,36) and *tppg* (Salk_078443,14,22) knockout mutants, the root epidermal cells were distributed similarly to the Wt with alternate trichoblast and atrichoblast files (data not shown). However, in the *tppa tppg* double mutant (Fig. 3B), the root epidermis consists of trichoblast files at some

![Figure 2](image)

Figure 2. Shoot phenotype of Wt Col-0 and *TPPB* mutant plants grown for 21 d on MS culture plates. (A) Rosettes of *tppb-1* and *tppb-2* mutants are significantly bigger than Wt Col-0 rosettes, at *p* < 0.001 (Student’s t-test, n = 6–10). In opposite, *TPPB-1* and *TPPB-2* overexpressors display significantly smaller rosettes compared with Wt Col-0, at *p* < 0.05 (Student’s t-test, n = 6–10). Numbers indicate the total leaf size ± SD. (B) Area of the individual rosette leaves shown in (A): Wt Col-0 (green bars), *tppb-1* (blue line), *tppb-2* (red line), *TPPB-1* (black line) and *TPPB-2* (orange line). Error bars represent averages ± SD (n = 6–10).

### Table 1. Abaxial epidermal analysis of the first pair of true leaves in 21-d-old *TPPB* mutants

| Genotype | Leaf area (mm²) | Epidermal cell size (µm²) | Estimated number of cells/leaf | Stomatal index |
|----------|----------------|---------------------------|-------------------------------|----------------|
| Wt Col-0 | 28.03 ± 2.52   | 1934 ± 191                | 24821 ± 3078                  | 21.6 ± 0.8     |
| *tppb-1* | 36.30 ± 4.67   | 1962 ± 127                | 33907 ± 4943                  | 22.6 ± 0.4     |
| *tppb-2* | 32.88 ± 3.62   | 2111 ± 141                | 28035 ± 1533                  | 22.8 ± 2.0     |
| *TPPB-1* | 21.83 ± 5.91   | 1819 ± 244                | 21709 ± 3660                  | 22.5 ± 2.2     |
| *TPPB-2* | 26.17 ± 5.91   | 1950 ± 136                | 24823 ± 4091                  | 22.8 ± 1.2     |

Values are averages ± SD (n = 6–10 images). Significant differences at, *p* < 0.05; *a* *p* < 0.01 (Student’s t-test).
Disruption of the TPPB gene leads to plants with larger leaves, which seems the result of an increased amount of epidermal cells. TPPA and TPPG proteins on the other hand, likely fulfill a redundant role during the development of atrichoblasts in the root epidermis. Altogether, these observations illustrate the non-redundancy and redundancy in function of the large TPP multi-gene family in Arabidopsis thaliana.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Nico Van Goethem for the assistance with preparation of the figures and Dr. Frantisek Baluska for the invitation to write this short communication. This research was supported by grants from the Fund for Scientific Research Flanders (FWO G.0859.10), the Industrial Research Fund of the KU Leuven (IOF/KP/08/001) and the VIB International PhD Program 2006.

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Figure 3. The role of TPPA and TPPG during the development of root epidermal cells in Arabidopsis seedlings. (A) Promoter TPP::GUS-GFP lines show TPPA and TPPG expression in atrichoblasts of root elongation and root meristematic zones, respectively. (B) The root of Wt Col-0 seedlings consists of files with trichoblasts and atrichoblasts, while the root epidermis of the tppa tppg double mutant is restricted to files with trichoblasts.
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