Trehalose-6-Phosphate Synthase/Phosphatase Regulates Cell Shape and Plant Architecture in Arabidopsis\textsuperscript{1}[W][OA]

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The vacuole occupies most of the volume of plant cells; thus, the tonoplast marker \(\delta\)-tonoplast intrinsic protein-green fluorescent protein delineates cell shape, for example, in epidermis. This permits rapid identification of mutants. Using this strategy, we identified the cell shape phenotype-1 (csp-1) mutant in Arabidopsis thaliana. Beyond an absence of lobes in pavement cells, phenotypes included reduced trichome branching, altered leaf serration and stem branching, and increased stomatal density. This result from a point mutation in \(\text{AtTPS6}\) encoding a conserved amino-terminal domain, thought to catalyze trehalose-6-phosphate synthesis and a carboxy-terminal phosphatase domain, is catalyzing a two-step conversion to trehalose. Expression of \(\text{AtTPS6}\) in the Saccharomyces cerevisiae mutants \(\text{tps1}\) (encoding a synthase domain) and \(\text{tps2}\) (encoding synthase and phosphatase domains) indicates that \(\text{AtTPS6}\) is an active trehalose synthase. \(\text{AtTPS6}\) fully complemented defects in \(\text{csp-1}\). Mutations in class I genes (\(\text{AtTPS1–AtTPS4}\)) indicate a role in regulating starch storage, resistance to drought, and inflorescence architecture. Class II genes (\(\text{AtTPS5–AtTPS11}\)) encode multifunctional enzymes having synthase and phosphatase activity. We show that class II \(\text{AtTPS6}\) regulates plant architecture, shape of epidermal pavement cells, and branching of trichomes. Thus, beyond a role in development, we demonstrate that the class II gene \(\text{AtTPS6}\) is important for controlling cellular morphogenesis.

Cell shape is an important component of tissue and organ development and morphogenesis in all living organisms and shape is acquired during the process of cellular differentiation. In plants, most cells are immobile and attached to one another and, with few exceptions, are cylindrical. However, more complex shapes are found and include lobed pavement cells in the leaf epidermis of some species and trichomes of leaves and stems. Trichomes have been the subject of investigations into mechanisms that underlie cell shape determination in plants (Qiu et al., 2002; Deeks and Hussey, 2003; Smith, 2003; Wasteneys and Galway, 2003; Schellmann and Hulskamp, 2005). Mutants in maize (\(\text{Zea mays}\)) and Arabidopsis (\(\text{Arabidopsis thaliana}\)) have pointed toward the cytoskeleton as an important determinant of cell shape (Smith et al., 1996; Qiu et al., 2002; Mathur et al., 2003; Mathur, 2004) paralleling similar discoveries in animal cells. In lobed leaf pavement cells, transversely arranged bundles of microtubules (MTs) in neck regions alternate with regions of cortical fine actin microfilaments where there are no MTs to promote the outgrowth of lobes (Frank and Smith, 2002; Fu et al., 2002). By screening for mutants in pavement cell shape using a GFP marker to readily visualize morphology, we describe here an example of a completely new class of mutants that affect cell shape: mutants in trehalose-6-P synthases (TPSs) / trehalose-6-P phosphatases (TPPs).

Trehalose is a nonreducing disaccharide composed of two Glc units that is present in diverse organisms, such as bacteria, fungi, lichens, algae, and invertebrates (Augier, 1954; Elbein, 1974; Goddijn and van Dun, 1999; Elbein et al., 2003). Initially, trehalose was believed to be present only in desiccation-tolerant plants, such as Selaginella lepidophylla and Myrothamnus flabellifolia (Adams et al., 1990; Müller et al., 1995); however, more recently it has been demonstrated to be at low levels in all plants. Furthermore, a yeast (\(\text{Saccharomyces cerevisiae}\)) loss-of-function mutant in \(\text{trehalose-6-phosphate synthase1}\) (\(\text{tps1}\)) is complemented by the Arabidopsis homolog \(\text{AtTPS1}\), indicating the presence of functionally equivalent biosynthetic genes in other plant species (Goddijn and Smeekens, 1998). In fact, all plants examined have the genes necessary for trehalose biosynthesis. Arabidopsis has 11 homologs encoding \(\text{TPS}\)s that are known to produce transcripts and are thus presumed to be functional. There is considerable evidence for a role of trehalose in protection from heat, osmotic, nutrient, and dehydration stress, as well as toxic chemicals. It may also play a protective role in the oxidative stress of proteins and lipids (Wiemken, 1990; Crowe et al., 1992; Newman et al., 1993; Fillinger et al.,

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and has been shown to function in carbohydrate storage (Elbien et al., 2003).

Beyond these more established roles of TPS genes in plants, recent intriguing evidence has implicated these genes as important modulators of plant development and inflorescence architecture. In one dramatic example, trehalose appears to modulate inflorescence branching in maize (Satoh-Nagasawa et al., 2006). Inflorescence branching in maize is controlled by the RAMOSA genes, and one of the genes (RAMOSA3) encodes a trehalose biosynthetic gene that functions through the regulation of the transcription factor RAMOSA1 (Satoh-Nagasawa et al., 2006). The results indicate that inflorescence architecture is controlled either by trehalose or, even more exciting, by a more direct transcriptional mechanism involving the protein. Other impacts on development by TPS genes have also been reported (see below).

The most common biosynthetic pathway for trehalose in plants is transfer of Glc from UDP-Glc to Glc-6-P resulting in trehalose-6-P and UDP. This initial step is catalyzed by TPS. In a second step, the dephosphorylation of trehalose-6-P occurs via TPP producing trehalose. In yeast, a large enzyme complex displaying both TPS and TPP catalytic activity is well described, and the ScTPS1 gene and trehalose have been shown to be involved in stress protection and control of sugar flux into the glycolytic pathway via inhibition of hexokinase II (Bonini et al., 2000; van Vaeck et al., 2001). A TPS1 yeast deletion mutant does not grow on Glc as a carbon source (Van et al., 1993), suggesting that trehalose can act as a signaling molecule. Among Arabidopsis homologs, only AtTPS1 is well characterized (Blázquez et al., 1998; Gómez et al., 2006). It is a class I TPS gene in that it possesses an N-terminal synthase domain but lacks the C-terminal phosphatase domain (Leyman et al., 2001). Expression of AtTPS1 has been linked to carbon metabolism and specific developmental phenotypes, such as delayed embryo development, altered root and shoot growth, and altered transition to flowering (Blázquez et al., 1998; van Dijken et al., 2004). Homozygous null mutants of AtTPS1 are embryo lethal, displaying cell wall thickening and altered morphology (Gómez et al., 2005, 2006). There is also evidence for a role in stress tolerance (Romero et al., 1997; Pilon-Smits et al., 1998).

In tobacco (Nicotiana tabacum), expression of Escherichia coli TPS (OTS A) results in increased photosynthetic activity, stunted growth, and lancet-shaped leaf morphology, whereas constitutive expression of TPP (OTS B) results in reduced photosynthesis (Paul et al., 2001; Pellny et al., 2004). Pleiotropic effects in growth and development have also been observed in Arabidopsis as a result of OTS A and OTS B expression (Schluempmann et al., 2003). The effects are not due to increased trehalose accumulation, but rather to an increase of the intermediate trehalose-6-P, which may act as an important signaling molecule (Jang et al., 2003; Schluempmann et al., 2003). More recently, expression of genes encoding TPS1 and TPS2 of yeast were found to affect root growth in Arabidopsis as well impart drought tolerance (Karim et al., 2008).

Here, we describe the identification and analysis of a cell shape phenotype-1 (csp-1) mutant that has a dramatic cellular effect in the leaf epidermis, resulting in loss of pavement cell lobes. In addition, csp-1 impacts the cell morphology of trichomes, resulting in an altered pattern of branching. The mutant shows a range of developmental defects that include reduced stature, altered stem branching, and pronounced leaf serrations. A mutation was found in the AtTPS6 gene. Significantly, we provide evidence indicating that this class II TPS gene functions in the control of cell morphology in addition to functioning as a broad modifier of whole-plant developmental phenotypes.

RESULTS

The csp-1 Mutant Displays Multiple Cellular and Developmental Phenotypes in Arabidopsis

Previously, we reported a microscopy-based screen to identify vacuole-defective mutants (Avila et al., 2003) using tonoplast marker GFP fused to δ-tonoplast intrinsic protein (35S:GFP::δ-TIP; Cutler et al., 2000). Laser-scanning confocal microscopy analysis of the cellular phenotype showed that the leaf pavement cells of the parental Arabidopsis line expressing GFP::δ-TIP were typical in having pronounced lobes resulting in a jigsaw puzzle-like appearance, whereas pavement cells of csp-1 showed either no lobes or lobes that were much less pronounced (Fig. 1, A and B). In addition, the loss of pavement cell lobes, the mutant displayed retarded development (see Fig. 2A), which in part may have accounted for a decrease in the size of individual cells in the hypocotyls compared to the parental line (Fig. 1, C and D). Root cells also appeared to be reduced in size (Fig. 1, E and F). Scanning electron micrographs of leaves of the csp-1 mutant and the parental line also clearly highlighted the altered pavement cell shape (Fig. 1, G and H). Interestingly, the surface morphology of the mutant cells was more deeply ridged in appearance compared to the parental line. A trichome branching phenotype was obvious in the mutant (Fig. 1, I–K). In the mutant, 98% of trichomes (n = 10 seedlings; four to six leaves per seedling for a total of 842 trichomes) displayed two branches compared to the parental line in which 100% of trichomes had three branches (n = six seedlings; one to three leaves per seedling for a total of 191 trichomes). Quantification of individual trichome branches of csp-1 indicated that, although the mutant produced fewer branches, they were longer than those of the parental line (Table I). The mutant displayed more stomates per unit area in csp-1 (mean of 295/ mm²; n = 5 seedlings; three to four leaves per seedling) compared to the parental line (mean of 227/ mm²; n = 5 seedlings; three to four leaves per seedling), indicating an impact on mechanisms controlling stomatal density.
Beyond changes in cellular morphology and stomatal density, the mutant displayed retarded development. The stature and morphology of both 10-d-old seedlings (Fig. 2A) and adult plants (Fig. 2, B and C) of *csp-1* were significantly different from the parental line. The mutant exhibited slow growth at the seedling stage and rosette leaves of the mutant were greatly reduced in size and narrower than the parental line, with pronounced serration (Fig. 2D). Mutant leaves displayed strong epinasty (downward folding; Fig. 2, C and D). At bolting, the mutant showed a greater number of primary shoots than the parental line, each of which displayed reduced branching and delayed flowering, suggesting a reduction in apical dominance (data not shown).

**Mapping of the csp-1 Locus via Microarrays**

To understand the basis of the cellular and developmental phenotypes associated with *csp-1*, we initiated a map-based cloning strategy to identify the locus and clone the mutated gene. The mutant in ecotype Columbia (Col) was crossed to Landsberg erecta (Ler) to obtain segregating populations for mapping. Individuals from the F2 mapping population were scored for the mutant *csp* via confocal microscopy. We utilized a high-throughput screening method for scoring and screened more than 5,000 recombinants (see “Materials and Methods”). In our mapping population, *csp-1* displayed segregation that was consistent with a recessive mutation (data not shown). To map the mutation, the ATH1 Arabidopsis expression array was used (Borevitz et al., 2003). The array contained 22,500 probe sets representing 24,000 genes. Based on hybridization to a pool of 100 F2 recombinants and differences in allelic frequencies (Borevitz et al., 2003), we predicted that *csp-1* resided on the lower arm of chromosome 1 between 9.11 and 12.09 Mb with the maximal frequency at 10.60 Mb (Supplemental Fig. S1). We observed a minor statistical peak on chromosome 4 between 8.14 and 9.8 Mb; however, we confirmed the position of the *csp-1* locus on chromosome 1 between the insertion-deletion markers nga280 (83.83 cM) and nga111 (115.55 cM). The position was also confirmed using simple sequence length polymorphism markers on chromosome 1 (Bell and Ecker, 1994; data not shown). The locus was located on bacterial artificial chromosome (BAC) T23K23, which was 101.966 kb in length and contained 38 genes (Fig. 3A). Sequence analyses of all 38 genes revealed a single missense

**Figure 1.** The *csp-1* mutant displays cell shape, size, and trichome aberrations. A to F, Confocal microscopy images of cells of 7-d-old seedlings expressing the tonoplast marker GFP::TIP. A and B, Leaf epidermal pavement cells of parental (A) and *csp-1* mutant lines (B). C and D, Hypocotyl cells of the parent (C) and mutant (D). E and F, Root cells of the parent (E) and mutant (F). G to K, Scanning electron microscopy images of similar 14-d-old seedlings. G and H, Leaf epidermal pavement cells of the parental line (G) and mutant (H). I, J, and K, Leaves of the *csp-1* mutant (left) and the parental line (right) display obvious differences in trichome biogenesis. J and K, Several individual trichomes are shown from *csp-1* (J) and the parental line (K), highlighting the differences in branch number. Arrows indicate positions of several epidermal cell lobes in the parent (A and G) and mutant (B and H). Bars = 50 μm (A and B); 150 μm (C–F); 100 μm (G and H); 500 μm (I); and 200 μm (J and K).
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Amino-terminal synthase and carboxyl-terminal phosphatase regions, class I and class II Arabidopsis TPS proteins fall into two distinct clades (Supplemental Fig. S3A). Gene expression of AtTPS6 was in all organs examined in Arabidopsis, including seedling, rosette leaf, cauline leaf, flower, root, and silique (Supplemental Fig. S3B). We also analyzed AtTPS6 mRNA accumulation in csp-1 and the transcript was detected in the mutant (Supplemental Fig. S3C), suggesting that the mutation altered protein function but did not alter gross gene expression.

AtTPS6 Plays an Important Role in Cell Shape and Encodes Functional Domains Involved in Trehalose Biosynthesis

Yeast TPS1 encodes a TPS, whereas TPS2 encodes a TPP. A tps1 mutant cannot grow on Glc as a carbon source because trehalose is necessary for the regulation of glycolysis by negatively regulating hexokinase II in vivo (Blázquez et al., 1993). A tps2 deletion mutant in yeast showed reduced trehalose accumulation and an accompanying increase in thermostensitivity (De Virgilio et al., 1993). Because AtTPS6 possessed both synthase and phosphatase domains, we hypothesized that it would complement both tps1 and tps2 deletion mutant phenotypes in yeast if fully functional. Indeed, AtTPS6 complemented the Glc sensitivity of the synthase-deficient mutant tps1 and thermosensitivity of the phosphatase-deficient mutant tps2 of yeast (Fig. 1B). These results indicate that AtTPS6 is a multifunctional enzyme encoding functional synthase and phosphatase domains.

The complete AtTPS6 coding region plus 5′ and 3′ regulatory regions (TPS6Pro::TPS6) was amplified using gene-specific primers from genomic DNA and cloned into a modified pBIN19-GW binary vector. The construct was used to transform csp-1 mutant plants for complementation (Clough and Bent, 1998). Several transgenic lines were isolated that were kanamycin resistant and displayed complete restoration of pavement cell morphology (Fig. 5A) when compared to csp-1 (Fig. 1B) and similar to the parental line (Fig. 1A).

Also restored were normal leaf shape, growth (Fig. 5, B and C), and typical trichomes with three branches (Fig. 5D). These results indicate that AtTPS6 complemented the cell shape defects (pavement cells, trichomes) and overall growth phenotypes displayed by the csp-1 mutant. We concluded that AtTPS6 plays a significant role in modulating cellular shape as well as growth and development in Arabidopsis.

### Table 1. Trichome branch length (μm)

All measurements ± SE; n = 15 trichomes for each measurement. For csp-1, branch 3 not observed. Parental line is Col-0 expressing GFP::δ-TIP.

<table>
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<tr>
<td>Parental</td>
<td>191.7 ± 11.7</td>
<td>154.5 ± 6.6</td>
<td>143.2 ± 11.2</td>
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<tr>
<td>csp-1</td>
<td>231.8 ± 13.6</td>
<td>223.5 ± 12.2</td>
<td>None</td>
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Disruption and Overexpression of AtTPS6 Dramatically Altered Cell and Developmental Phenotypes

A T-DNA insertion mutant (Alonso et al., 2003) from the SALK collection (csp-2) was characterized at the molecular and phenotypic levels. Genotyping of csp-2 using the left border primer LbB1 and a gene-specific reverse primer indicated the position of the insertion in AtTPS6 (Fig. 3B). The T-DNA insertion was in the exon at position 9 nucleotides within the synthase domain and abolished AtTPS6 mRNA accumulation as evidenced by semiquantitative RT-PCR analysis (data not shown). Pavement cells were stained with FM4-64. This dye, which stains the plasma membrane, endomembranes, and vacuole, will also bind to the cell wall conveniently outlining cell shape. This staining revealed a cell shape defect in csp-2 similar to that observed in csp-1 (Fig. 6A; compare to Fig. 1B). Interestingly, the csp-2 loss-of-function mutant did not display other developmental leaf phenotypes, such as pronounced serration, epinasty, and reduced trichome branching, as observed in csp-1 (Fig. 6B; compare to Fig. 2C). This indicated that control of cell shape was separable genetically from developmental defects such as leaf morphology and expression of the mutated gene (csp-1) and the complete loss of function (csp-2) resulted in cell shape defects. Loss-of-function mutants in the remaining class II AtTPS genes AtTPS5, AtTPS7, AtTPS8, AtTPS9, and AtTPS10 were also stained with FM4-64 and revealed no cell shape defects as observed in the AtTPS6 mutants csp-1 and csp-2 (data not shown). These results indicate that AtTPS6 is unique among class II AtTPS genes in affecting the cell shape of leaf pavement cells.

To further investigate the role of AtTPS6 in development, the coding region of AtTPS6 was overexpressed in Arabidopsis under the control of the constitutive 35S promoter. The overexpression lines displayed an increase in AtTPS6 transcript as detected by multiplex semiquantitative RT-PCR (data not shown). The phenotypes displayed by eight independent lines overexpressing AtTPS6 were striking and related to those displayed by csp-1. For example, at 3 weeks old, the rosette leaves and overall stature of the plants were significantly greater than those of the parental line (Figs. 6C and 2B, respectively). In addition to these developmental phenotypes, the trichomes of all overexpression lines displayed an increased number of trichome branches, ranging from four to six compared to the parent, which displayed three branches (Figs. 6D and 1K, respectively).

The independent lines displayed a range of related phenotypes correlated with their levels of AtTPS6 expression. We classified these lines into two general categories. For lines in category 1, the greatest amount of transcript was detected (Fig. 6F, inset), as well as a dramatic increase in the overall size and number of rosette leaves (Fig. 6E) and fewer bolts, suggesting an increase in apical dominance. Among category 2 overexpression lines, there was a more modest increase in expression (Fig. 6F, inset), resulting in fewer leaves, each having reduced serration compared to category 1 mutants. Overall growth was also slower than that of the category 1 lines (Fig. 6F). There was an apparent reduction in apical dominance as evidenced by increased primary shoots emerging from the rosette. There was also an increased number of flowering branches with altered leaf phyllotaxy compared to the parental line (data not shown). Overall, these results indicate that AtTPS6 plays a role not only in establishing cellular morphology, but also in the overall growth and morphology of the organism.

DISCUSSION

We have shown that the CSP gene plays a critical role in the regulation of cell morphogenesis and other developmental processes. Interestingly, CSP encodes the multifunctional enzyme AtTPS6, suggesting that sugars are capable of regulating cell morphogenesis in plants, a process that is dependent upon signal-mediated...
cytoskeletal dynamics and reorganization (Smith and Oppenheimer, 2005). Although the cytoskeleton has been shown to influence plant sensitivity to sugar responses, our results demonstrate that a class II TPS/TPP enzyme involved in sugar catabolism has a role in the control of cell morphogenesis. We have also found that CSP modulates a multitude of other developmental processes, such as plant stature, leaf morphology, shoot apical dominance, and root development. Overall, our findings support a concept in which AtTPS6 is an important regulator of plant development via a role in generating a sugar-based signal or more directly by participating in transcriptional regulation.

**AtTPS6 Is Functionally Equivalent to TPS and TPP of Yeast**

The TPS proteins from organisms as distant as bacteria, yeast, and plants are highly conserved at both the structural and functional levels; these similarities suggest evolutionarily conserved functions. Among the 11 Arabidopsis TPS genes, only AtTPS1, a class I gene possessing the synthase domain alone, has been investigated functionally (Leyman et al., 2001). Previous studies indicated that AtTPS7 and AtTPS8 may be inactive enzymatically in Arabidopsis (Vogel et al., 2001). Thus, we asked whether AtTPS6 was active functionally. Our data show that the gene is expressed in all organs examined in Arabidopsis, arguing that the gene is functional and ubiquitous. Furthermore, because AtTPS6 possesses both synthase and phosphatase domains, we predicted that the gene would complement yeast deletion mutants of both tps1 (synthase domain only) and tps2 (phosphatase domain only). Validation of this prediction strongly indicates that AtTPS6 is an active class II bifunctional enzyme.

![Figure 4. AtTPS6 has functional synthase and phosphatase domains.](image)

A, Schematic showing protein domains of TPS1 and TPS2 from yeast ScTPS1 and ScTPS2 and their homologs in Arabidopsis AtTPS1 and AtTPS6, respectively. B, Complementation of yeast with corresponding Arabidopsis genes. Top, As a control, wild-type yeast (wild type), a ScTPS1 deletion mutant (Δtps1), and the deletion mutant expressing AtTS6 (Δtps1 + AtTPS6) grew on Gal as a carbon source. When grown on Glc, wild-type yeast grew normally, whereas the deletion mutant (Δtps1) could not utilize Glc as a carbon source. When AtTPS6 was expressed in Δtps1 (AtTPS6 + Δtps1), yeast grew vigorously, indicating that AtTPS6 complemented Δtps1 functionally. Bottom, When grown at the permissive temperature (28°C), wild-type yeast, a deletion mutant (Δtps2), and AtTPS6 expressed in Δtps2 (Δtps2 + AtTPS6) all grew normally. However, at the nonpermissive temperature, Δtps2 could not grow, whereas the strain expressing AtTPS6 (Δtps2 + AtTPS6) displayed growth similar to that of the control (wild type), indicating functional complementation by AtTPS6.
TPS6 Is a Class II Enzyme That Controls Cellular Morphology and Modifies Growth and Development

We found that the mutation in csp-1 conferred pavement and trichome cell shape defects in addition to several strong developmental phenotypes, including altered leaf size and morphology, reduced plant stature, and increased stem branching. These phenotypes indicate that AtTPS6 plays an important role not only in controlling cellular morphogenesis, but also in modifying overall development. Interestingly, it was reported that the Arabidopsis class I tps1 mutant displays defects including embryo lethality and an effect on the transition to flowering (van Dijken et al., 2004). However, these phenotypes are different from those of csp-1. In any event, there are no reports to date describing developmental defects associated with class II TPS members, much less dramatic alterations in cell shape.

Among the cellular morphology phenotypes in csp-1, we observed two-branched trichomes. In Arabidopsis, there are at least 24 genes known to regulate trichome morphogenesis and branching (Oppenheimer, 1998) and there may be several redundant pathways. One of the branching mutants is regulated by the transcription factor basic helix-loop-helix that affects endoreduplication (Oppenheimer, 1998). However, there is also an endoreduplication independent pathway in trichome branch patterning. This work describes the involvement of TPS genes in trichome development. Complementation studies of the point mutant line csp-1 demonstrated the restoration of all cellular and developmental defects, indicating that the synthase domain where the mutation resides is critical for modifying development and cellular morphology, including that of trichomes. This is further substantiated by the finding that overexpression of AtTPS6 in Arabidopsis results in increased trichome branching as well as the restoration of typical pavement cell shape.

Role of AtTPS6 in Determining Cell Shape

Key components of cell shape are F-actin and MTs, which are important determinants of cell polarity and cell wall remodeling. Accordingly, the three known classes of genes known to participate in cell morphogenesis in plants are (1) cytoskeletal proteins and proteins regulating the cytoskeleton, (2) proteins regulating polarized secretion, and (3) proteins involved...
in cell wall synthesis or remodeling. Many mutants in Arabidopsis that are defective in the morphogenesis of both pavement cells and trichomes are affected in either F-actin or MTs. Loss of function of components of the Arp2/3 actin-nucleating complex causes reduced lobe expansion in pavement cells and distorted trichomes that display swollen stalks and retarded branch expansion (Mathur, 2005). Defects in the stability or order of MTs result in distinct phenotypes such as reduced trichome branching and waviness of the jigsaw puzzle-shaped pavement cells by increasing expansion of the sinus region (Fu et al., 2002, 2005; Mathur, 2005). In contrast, increased stability or ordering of MTs results in increased branching of trichomes and inhibition of lobe formation in pavement cells (Fu et al., 2002, 2005; Li et al., 2003; Mathur et al., 2003; Mathur, 2005). Interestingly, from our analysis of both loss-of-function and overexpression phenotypes, we demonstrated that AtTPS6, which possesses both TPS and TPP functions, is involved in the control of pavement cell shape and trichome branching. This is reminiscent of defects in MT organization and future investigations can address this.

Potential interaction between sugar metabolism and actin organization and dynamics is suggested by ARF2/3-defective mutants that have altered responses to sugars (Li et al., 2003). Our findings show that a sugar catabolic enzyme can have an influence on a cytoskeleton-mediated process and suggest an interaction between sugar metabolism and cytoskeletal organization and dynamics.

The obvious question that arises is how AtTPS6 could be involved in the determination of cell shape. One possibility is that trehalose-6-P or a related metabolite could serve as a signal in controlling the MT organization necessary for cell shape determination as has been demonstrated for the morphogenesis of pavement cells (Fu et al., 2002, 2005). Although speculative, it should be possible to test this hypothesis by investigating the effect of trehalose-6-P or csp-1 and csp-2 on ROP GTPase signaling during the formation of pavement cell lobes (Fu et al., 2002, 2005). Another possibility is that AtTPS6 could be involved directly in the transcriptional regulation of genes encoding proteins involved in cytoskeletal organization (see below). This possibility is consistent with our observation that the cell shape phenotypes were not reported for AtTPS1 (Blázquez et al., 1998; van Dijken et al., 2004) or other the class II AtTPS genes that we examined. This transcriptional hypothesis is also consistent with our findings that csp-1 and csp-2 had pleiotropic effects on developmental processes.

Role of AtTPS6 in Development

Several previous reports have suggested that trehalose-6-P may be acting as a regulatory molecule involved in metabolism and embryo development (Eastmond et al., 2002). Three different tps1 T-DNA alleles are embryo lethal, show reduced root growth, and affect the transition to flowering (van Dijken et al., 2004). Interestingly, these phenotypes were not seen with csp-2. One explanation may be related to the tissue-specific expression profile of the genes. Whereas AtTPS6 is expressed ubiquitously, AtTPS1 is expressed at low levels and concentrated in the flower buds, siliques, and young rosettes (van Dijken et al., 2004). The more ubiquitous expression profile of AtTPS6 may also explain its broader effects on development and suggest that this gene may play a more central role in modifying development. Conversely, AtTPS1 may play a more specialized role in modifying embryo or flower development.

The csp-1 point mutation in the synthase domain of AtTPS6 results in cell shape, leaf, and branching phenotypes. It also imparts a drought-tolerant phenotype (S.N. Chary and N.V. Raikhel, unpublished data). Although we do not know at this time whether the AtTPS6 protein is present in vivo, the mutant displays detectable gene expression, indicating that the point mutation may be affecting these plant developmental phenotypes within the context of altered protein function. The T-DNA null allele csp-2 results in the pavement cell shape defect only and not in developmental phenotypes observed in csp-1. This indicates that the point mutation in csp-1 alters a synthase domain function necessary for modulating development (in addition to cell shape). The cell shape phenotype is more mysterious. Although we do not fully understand the basis for the developmental phenotypes in the null and point mutants, one speculation is that the null mutant csp-2 does not cause developmental defects because there is no phosphatase domain that may be important for the developmental phenotypes. In the point mutant csp-1, the interaction of the altered synthase domain, plus an active phosphatase domain, may be necessary for the developmental phenotype. It is also conceivable that the null mutant fails to display developmental defects due to gene redundancy. Overall, this hints at the complex role that AtTPS6 may play in plant development.

There is recent evidence that the maize protein RAMOSA3 (TPP) affects the development of inflorescence branching via possible interaction with RAMOSA1 (a predicted transcriptional regulator). However, there is no evidence for interaction between the transcription machinery and AtTPS6. In the case of csp-1, the ethyl methanesulfonate mutation confers a recessive phenotype, suggesting a loss of function that results in altered cell shape, decreased trichome branching, slower growth, and altered leaf morphology. This is supported by the null mutant csp-2, which displayed the cellular phenotype (but not the developmental phenotypes). Furthermore, the increase in rosette leaf size and trichome branching among overexpression lines suggests that AtTPS6 acts in a positive manner to affect development. This notion is also supported by the fact that increased expression of AtTPS6 among category I overexpression mutants results in an apparent increase in apical dominance. The observation...
that mild overexpression (category II) results in reduced apical dominance similar to that observed in csp-1 cannot be explained fully at this time. However, it indicates that the processes affected by the mutated gene and mild overexpression are impacting similar developmental pathways.

MATERIALS AND METHODS

Plant Materials and Plant Growth

T-DNA insertion mutant seed was obtained from the Arabidopsis Biological Resource Center. Arabidopsis (Arabidopsis thaliana) ecotype Col, csp-1 mutant (35S-3TP1-GFP in Col background), and other mutants were grown on one-half-strength Murashige and Skoog medium containing 1% Suc and 0.8% phytoagar. Plates were placed in a 4°C chamber for 2 d in the dark for vernalization and allowed to germinate and grow at 22°C under long-day conditions (18 h light/6 h dark). After 7 d, seedlings were transplanted to Promix and grown under the same long-day conditions. For the F2 segregating population, seeds were germinated on thin Universal lids (Costar) on one-half-strength Murashige and Skoog medium with 1% Suc and 0.6% phytoagar and grown further at 22°C under long-day conditions in a growth chamber with the seedlings positioned vertically. For all crosses, floral buds of csp-1 (Col background) were dissected and pollinated using mature pollen from Ler flowers.

Plant Transformation and Positional Cloning

Arabidopsis ecotype Col plants were transformed with all constructs using Agrobacterium tumefaciens GV3101 (pMP90) by the floral-dip method (Clough and Bent, 1998). The csp-1 mutant was crossed to Ler ecotype. F1 heterozygotes from this cross were self-fertilized to generate an F2 mapping population. F2 recombinants were screened on an automated BD-Biosciences Pathway confocal microscope.

Expression Array-Based Mapping

Leaf tissue of equal amounts was collected from each of 100 F2 mutant and wild-type plants from a segregating population using a leaf punch and frozen in liquid nitrogen. Tissues were ground to a fine powder and genomic DNA was isolated using DNAzol (Invitrogen). Three hundred nanograms of genomic DNA from each pool were randomly biotin labeled by using the Bioprime DNA labeling system (Invitrogen). The fragment-labeled DNA (approximately 25–50 bp) was purified using a Qiagen genomic DNA purification kit according to manufacturer. One-step RT-PCR was performed using Universal 18S rRNA primers in a 2:8 ratio of 18S rRNA primers to competitors (Ambion). The template RNA for 18S rRNA control reactions consisted of 0.5 μg of total RNA from mouse liver (Ambion). RT was performed at 50°C for 20 min, after which PCR was performed in a total reaction volume of 50 μL using PCR cycling conditions of 95°C, 5 min; 94°C, 15 s; 65°C, 30 s; 72°C, 1 min; and 72°C, 4 min for 32 cycles.

DNA Constructs for Complementation and Overexpression

AtTPS6 was PCR amplified from genomic DNA of Arabidopsis ecotype Col using the following primers: TPS6F, 5′-GCTCGATCCACCTAGCTTCGT-GCCCTTTGCGGTATCAG3′; and TPS6R, 5′-CGTAGAGAAACCAACAGG-GAAACAGGAGTGATCTGGTCTG-3′. The amplified PCR fragment was cloned into the pDONOR207 vector after confirming the sequence; the insert was integrated into the pBIN-GW binary vector and used to transform csp-1 as described above. T1 seeds were germinated on Murashige and Skoog with 50 μg/ml kanamycin. Km-resistant seedlings were transferred to liquid pots and propagated to T2 generation. Selected transgenic lines resistant to kanamycin were grown to the T3 generation to obtain homozygous lines.

The AtTPS6 coding region was PCR amplified using gene-specific primers from genomic DNA of Arabidopsis ecotype Col. Primers were TPS6f-LumHIL, 5′-CAGCTGGATCCATGGTTTCAAGATCGTATTCAAATCTG-3′; and TPS6-R-XhoI, 5′-TCCTCCTCGATAGCATGTGAGAAAGA3′. The amplified PCR fragment was digested with appropriate restriction enzymes and purified and cloned into the modiﬁed plant binary vector pZip11 containing a 35S promoter with a translational enhancer (Ω-leader sequence). The construct was transformed and independent kanamycin-resistant lines were isolated.

Yeast Expression Constructs

The coding region of AtTPS6 was PCR amplified from Arabidopsis genomic DNA using a primer with 5′ Kpn I and 3′ XhoI restriction sites for compatible restriction sites for cloning into the yeast (Saccharomyces cerevisiae) expression vector pYES2. The primers were: TPS6-KpnI, 5′-GGGGGTAACATGAGCCGAGAAGCTGCATGACTATCTGCTGGAATCT-3′; and TPS6-XhoI, 5′-GGGGTCAACCGTATGCTAATCCGTTGCTGCGTGCATGTTCGACGC-3′. PCR-ampliﬁed products were digested with Kpn I and XhoI and ligated to KpnI-XhoI-digested pYES2. The AtTPS6 construct in pYES2 was used to transform the yeast tps1 and tps2 deletion mutants (OpenBioSystems).

Confocal Microscopy

Ten-day-old seedlings of the parental line, mutants, and overexpression lines were examined on a Leica SP2 confocal microscope, using 20×/0.7NA and 63×/1.2NA HC PL APO water objectives. The 488-nm argon laser line was used for excitation of GFP and emission was collected between 500 and 600 nm. FM4-64 was excited at 488 nm and detected between 600 and 750 nm. For mapping, the BD Biosciences Pathway HT imaging system was used with a UAF0/340 20×/0.75NA objective and custom Relflector wheels containing the Semrock Brightline GFP-303SB filter set with 472/30-nm excitation and 520/35-nm emission.

Scanning Electron Microscopy

Parental and mutant leaves were flash frozen by immersion in 1,1-difluoroethane in a liquid nitrogen-cooled mortar, then immediately transferred to the scanning electron microscope vacuum chamber for scanning at 15 keV using back-scattered electron detection on a Hitachi TM-1000 tabletop scanning electron microscope.

Morphometric Analysis

Trichome branch lengths were measured from scanning electron micrographs using Imaging Research MCID Elite software. The lengths and widths...
of parental and csp-1 mutant hypocotyl cells were tabulated from cells using 20× Leica confocal micrographs. For the parental line, 168 cells were examined and for the csp-1 mutant 209 cells were examined from independent seedlings.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At1g68020 (AtTPS6), csp-2 (SALK T-DNA insertion mutant SALK_105965.55.25.x).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Mapping of the csp-1 locus using expression array bulk segregant analysis.

Supplemental Figure S2. Multiple alignments of AtTPS family members in Arabidopsis.

Supplemental Figure S3. Class I and class II TPSs in Arabidopsis are distinct.

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of photosynthesis with *E. coli* genes for trehalose synthesis. Plant Biotechnol J 2: 71–82


