Chapter 4

Analysis of expression of trehalose-6-phosphate synthase/phosphatase and nucleotide binding protein in *Arabidopsis* using promoter:*GUS* fusions

Introduction

Differential display analysis revealed two genes that showed differential regulation in roots of *Cypripedium parviflorum* var. *pubescens* (CyPP) in response to a mycorrhizal fungus: trehalose-6-phosphate synthase/phosphatase (*Tps*) and nucleotide binding protein (*NuBP*). Further analysis revealed that, in orchid, *Tps* showed down-regulation in response to the fungus at later stages of plant growth and up-regulation at earlier stages of growth. It was also repressed by trehalose and induced by sucrose. Orchid *NuBP* was up-regulated by treatment of plants with the fungus and also by trehalose. The intractability of orchid as a model to study gene function means that another system must be used. *Arabidopsis* makes an excellent model because of its short life cycle, ease of transformation and the fact that the genome has been sequenced. Analysis of the heterologous genes in *Arabidopsis* (*AtTps* and *AtNuBP*) was performed to further study the function of *NuBP* and *Tps.*

Analysis of the homolog of CyPP *Tps* in *Arabidopsis* has not been undertaken. However other genes in the trehalose synthesis pathway have been analyzed and computer biology has revealed 11 homologs of *AtTps* (Leyman et al., 2001). These 11 homologs can be divided into two classes based on their homology to yeast *AtTps*: class I has high homology to yeast *Tps1* and is believed to catalyze the first step in trehalose synthesis; class II has higher homology to yeast *Tps2* which catalyzes the removal of the phosphate group to produce trehalose. Yeast *Tps2* is believed to form a complex with *Tps1* through interaction of the N-terminal half of both proteins (Londesborough and Vuorio, 1991). This has also been proposed in *Arabidopsis*, since the class II *AtTps* genes cannot rescue yeast mutants deficient in *Tps1* suggesting that the N-terminal synthase domain is inactive (Leyman et al., 2001). However, they are unable to complement yeast mutants deficient in *Tps2*. There are specific trehalose-6-phosphate phosphatase genes in *Arabidopsis* that are able to complement the yeast *Tps2* mutant (Vogel et al., 1998), which raises questions regarding the class II *Tps* homologs of *Arabidopsis*.

The large number of trehalose synthesizing genes and especially of the class II genes has led to the proposal that trehalose or treahlose-6-phosphate is a regulatory compound within the plant interacting with sugar sensing and signaling pathways (Leyman et al., 2001). The fact that multiple genes exist suggests that trehalose or trehalose-6-phosphate is tightly regulated and that this regulation may be in an organ or stimulus dependent manner. Since genome analysis has revealed that other plants have multiple copies of trehalose synthesizing genes, it is likely that trehalose is a regulatory

signal or compound in a diverse array of plants. Analysis of the *Arabidopsis* genes encoding *Tps* homologs will have implications on trehalose metabolism in other species.

Though a member of the *MinD* gene family has been identified and characterized in *Arabidopsis*, it does not seem to be of the same type as that identified in orchid. The AtMinD1 protein is targeted to chloroplasts where it is involved in chloroplast division (Colletti et al., 2000). The *Arabidopsis* homolog to the deduced amino acid sequence CyPP NuBP (AtNuBP) does not have a signal sequence and protein analysis suggests that it is a cytosolic protein. Further it only shares 28% identity with AtMinD1. Though the overall identity is low, the identity in certain regions is very high suggesting that AtNuBP has a similar function to that of AtMinD1.

Promoter:*GUS* fusions were created in *Arabidopsis* to investigate the expression pattern of *AtNuBP* and *AtTps*. These were subjected to various stimuli to determine when and where each gene was active in *Arabidopsis*.

Materials and Methods

Plant Material

All plants used were *Arabidopsis thaliana* ecotype Columbia. Seed was planted in 10 cm pots of synthetic potting soil (Sunshine Mix, Wetsel, Harrisonburg, Virginia), watered and kept at 4°C for 2 d. The pots were then placed on a potting stand composed of two, cool-white fluorescent lights suspended 30 cm above a plastic shelf, at room temperature. Plants were maintained at room temperature under lights. They were watered as needed and fertilized every other watering with a half-strength Hoagland's solution.

Genomic DNA Isolation

Genomic DNA isolation was performed as described in Chapter 3. DNA was isolated from 1 g of young *Arabidopsis* leaves.

Cloning of Promoters

Homologs of CyPP *Tps* and *NuBP* were identified to clone the promoters from *Arabidopsis* through BLASTn searches at the National Center for Biotechnolgy Information (NCBI) website (http://www.ncbi.nlm.nih.gov) and sequences adjacent to the start site of both genes were identified in *Arabidopsis*. For *AtTps*, approximately 1600 bp of sequence upstream of the putative transcription start site was selected from the highest nucleotide match to the initial BLASTn search (AC003671.1). For *AtNuBP*, approximately 600 bp of sequence from the stop codon of the previous gene to the initiation codon of *AtNuBP* (AB017063) was selected as the promoter. Primers were designed to amplify these regions through PCR (*AtTps* Fwd 5' CACTTTGTAGAGCTCAGACTC; *AtTps* Rev 5' GTCATGACGCGTGGAAGAG;

AtNuBP Fwd 5' GGATTTGTTCTAGAGTCATG; *AtNuBP* Rev 5' GACTAGTCGACTTGAGACTTCTCGAAG). PCR was performed on *Arabidopsis* genomic DNA with an annealing temperature of 55˚C for 37 cycles with an extension time of 1 min for *AtNuBP* promoter and 1.5 min for *AtTps* promoter. PCR products were cloned into pGEM T easy and sequenced at the Virginia Bioinformatics Institute Sequencing Facility. The sequences were checked against those in GenBank using BLAST to assure correctness of the clone.

*Creation of Promoter:*GUS *Fusion Vectors*

The binary vector pBI121 (Jefferson et al., 1987) was selected for insertion of the promoter next to *GUS* and for subsequent transfection into *Arabidopsis*. The binary vector was digested with *HindIII* restriction enzyme for 3 h at 37˚C. Klenow (10 units) and 500 µM dNTPS were added and the mixture was kept at 37˚C for an additional 15 min to blunt end the digestion. After heat killing the enzymes for 15 min at 70˚C, the mixture was extracted with chloroform, and DNA was precipitated with one tenth volume 7.5 M ammonium acetate and 2 volumes 100% ethanol. The pellet was resuspended in water and digested with *XbaI* restriction enzyme. This resulted in liberation of the 35S promoter from pBI121. The *AtTps* promoter in pGEM T easy was digested with *SpeI*, followed by blunt ending and extraction as above. The second digestion was performed with *XbaI*. The *AtNuBP* promoter was digested with *XbaI* followed by end filling and extraction as above. The second digestion was with *SpeI*, which leaves the same sticky end as *XbaI*. Digestion products were separated on a 0.8% low melt agarose gel and cut from the gel. The bands were melted at 65˚C for 10 min and then transferred to 37˚C. Digested promoter was mixed with digested vector in a 2:1 ratio and ligation buffer was added to 1x. Three to six units of T4 DNA ligase were added and the mixture was incubated at 37˚C for 1-2 h. The mixture was then cooled to 16˚C and maintained at that temperature overnight. The reaction was then brought to room temperature and kept there until use. The entire ligation reaction was used to transform 100 µl of *E. coli* competent cells (DH5α) which were spread on LB (see below) plates containing 50 µg/ml kanamycin (kan). Positive clones, which grew successfully on kan plates, were screened by PCR followed by restriction digests.

Transformation of Agrobacterium

Clones showing positive for pBI121 with the appropriate promoter were grown overnight in terrific broth (see below) DNA was extracted. DNA (1µg) was used to transfect 200 µl of competent *Agrobacterium* strain GV3101. DNA was added to cells and the reaction was placed on ice for 30 min. The mixture was fast frozen in liquid nitrogen for approximately 30 sec. This was then transferred to a 37˚C waterbath for 2 min until the cells had thawed. To this was added 800 µl of 2x TY and the cells were incubated at 28˚C with vigorous shaking for 2 h. 100 µl of this was plated on LB plates with rifampicin (34 µg/ml), kanamycin (50 µg/ml) and gentamycin (25 µg/ml). A second plate was prepared after the cells had been pelleted and resuspended in 100 µl 2x TY. Plates were incubated for 2 days at 28˚C.

In Planta Transformation of Arabidopsis

The method of Bechtold and Pelletier (1998) was used for in planta transformation. Six days prior to infiltration of *Arabidopsis* plants, developing inflorescences were cut to induce secondary bolting. Immediately prior to infiltration, developing siliques, open flowers and partially opened buds were cut from developing inflorescences. This assured that only closed, un-pollinated flower buds were used for transformation. Two days before infiltration, transformed *Agrobacterium* was used to inoculate 25 ml of 2x TY (see below) with rifampycin, gentamycin and kan. This was grown overnight and then added to 400 ml of fresh 2x TY with the same antibiotics. After 24 h of growth, cells were pelleted at 5000 RPM for 10 min. The supernatant was discarded and the cells were re-suspended in 500 ml of 5% sucrose. Surfactant, L-77, was added at 45 µl per 100 ml of cells. The cell suspension was poured into the lid of a pipette tip box and the *Arabidopsis* plants were supported upside down on the lid with their inflorescences splayed in the cell mixture. Plants were placed in a vacuum oven with 15 in Hg of vacuum applied for 15 min. Vacuum was released quickly and plants were wrapped in plastic film (Saran Wrap) and kept in the dark for 24 h. Plants were then returned to normal growing conditions. Seed was harvested when physiologically mature as determined by color change from green to brown.

Selection of Transformants

Seed of the T1 generation was sown in flats of synthetic potting media and covered with vermiculite. These were placed at 4°C for 2 days and then placed under lights at room temperature. As soon as seedlings emerged, seedlings were sprayed with kanamycin 50µg/ml every other day for four sprayings. Some seedlings died whereas others had white new leaves, which were removed. Surviving seedlings were analyzed for *GUS* expression using standard *GUS* staining protocols (see below). Plants showing positive for *GUS* were tagged and allowed to set seed. Seed was harvested and used for all remaining experiments.

Promoter Functional Analysis

T2 generation promoter:*GUS* lines were used for all subsequent experiments. Seed was sown on germination medium (GM, see below) with 12.5 mM sucrose and allowed to germinate in a growth chamber at 22°C, 16 h light 8 h dark cycle. After development of true leaves, seedlings were transferred aseptically to GM plates containing either 25 mM sucrose, 25 mM trehalose, 25 mM mannitol, or on to oatmeal agar plates with or without *T. pennatus*. After transfer, seedlings were grown on for 72 h and then used for *GUS* analysis following standard *GUS* staining protocols.

Media

LB, Terrific Broth and 2x TY media were prepared as described in Sambrook et al., (1989, Table 4-1). For liquid LB, the media was equilibrated to pH=7 and autoclaved. For solid LB, 15 g agar was added after pH adjustment and the mixture was

autoclaved followed by cooling to 60°C. Antibiotics were then added, the medium was poured into 150 mm diam. petri plates (Fisher) and allowed to set. Terrific broth was autoclaved prior to use. For 2x TY plates, agar was added to the liquid medium before autoclaving. After the medium was sterilized, it was allowed to cool, antibiotics were added and the media poured into 150 mm diam. petri plates. Germination medium (GM) was made according to the information at http://www.arabidopsis.org/weedsworld/Vol1/appendix1 (see also Sijmons et al., 1991). Media was prepared without sucrose and divided into 250 ml volumes. Sugars were then added to the appropriate concentration as was Bacto-agar (2 g/250 ml). The media were then autoclaved, cooled and poured into 150 mm (diam) petri plates.

GUS *Staining*

The *GUS* staining solution consisted of 50 mM $NaHPO_4$ (pH=7.2), diluted from a 100 mM stock), 0.5% Triton X-100, 1 mM X-Gluc (diluted from a 20 mM stock, 17.8 mg/2 ml dimethyl formamide). Organs to be examined for *GUS* were cut from the plant and immersed in 100 to 500 µl *GUS* staining solution in 1.5 ml microfuge tubes. The microfuge tubes were then placed in a speed-vac and a vacuum was pulled. Air could be seen escaping the tissue in the tube. The vacuum was pulled and released several times to assure infiltration of the *GUS* staining solution. Samples were incubated at 37°C for 24-48 h. The *GUS* staining was normally evident after 24 h and *GUS* staining solution was then removed and replaced with 200-500 µl 70% ethanol. Samples were analyzed under a dissecting microscope and photographed with a Nikon camera using Kodak T64 Ektachrome film.

Results

*Creation of Promoter:*GUS *fusion*

Promoters of both *AtNuBP* and *AtTps* were successfully cloned from *Arabidopsis* genomic DNA using PCR and sequencing revealed that both sequences were the ones selected. The promoters were then sub-cloned into pBI121 replacing the 35S CMV promoter (Fig. 4-1) and this was confirmed by restriction digest and PCR (Fig. 4-2 and 4-3). Transformation of *Agrobacterium* with promoter:*GUS* constructs produced approximately 50 colonies on selection medium. Two clones were kept as glycerol stock with one being used for plant transformation. Plant transformation efficiency was approximately 1% with three lines showing positive for *GUS* out of 3-400 seedlings with the *AtTps* promoter. Only two of the three lines showed positive for *AtTps* promoter:*GUS* fusions as analyzed by PCR (Fig. 4-4). To date, only one *AtNuBP* promoter:*GUS* plant has shown apparent *GUS* expression, which has not been confirmed.

Organ Level Expression

The *AtTps* promoter fusions showed expression in roots and cotyledons of seedlings (Fig. 4-5A) with strong expression at the root:hypocotyl junction. Expression

was also evident in developing root hairs. In older plants, there was no expression in leaves or stems (Fig. 4-5C). There was a strong wound response with *GUS* expression evident at sights of leaf removal or where stems were cut (Fig. 4-6). Roots continued to show expression in older plants (Fig. 4-5B), though expression seemed weaker with blue staining being less intense. Developing flowers also showed expression, which was evident only in the 5th flower from the apex and was concentrated in anthers and on the stigma (Fig. 4-7A). This expression was absent in flowers that had been pollinated though there was expression in the pedicel:silique junction (Fig. 4-7B). There was no expression in developing seeds. The expression of *GUS* in the *AtNuBP* promoter fusion plant is in developing pollen only (Fig. 4-9).

Expression in Response to Sugars and Fungi

Plants transformed with the *AtTps*:promoter fusions showed no qualitative changes in *GUS* expression in response to any of the sugars or fungus tested (Fig. 4-8).

Discussion

The analysis of *Arabidopsis AtTps* promoter represents the first time this gene has been characterized in *Arabidopsis*. While *AtTps1* has been studied through mutant analysis and by functional complementation of yeast *tps1*, it is a class I *Tps* gene whereas the gene studied here is a class II. The function of the class II *AtTps* genes is unknown since two class II *AtTps* genes clones into yeast failed to complement mutations in either *tps1* or *tps2*. The promoter of *AtTps* is active in *Arabidopsis* as seen by *GUS* activity. Though this does not necessarily reflect the synthesis of functional protein, it can be used to determine what triggers expression of *AtTps* and provides a basis for understanding gene function. Furthermore, promoter regions are not always upstream of transcription initiation start sites and it is possible that the regions of genomic DNA cloned as promoters are actually promoters of other genes.

In plants grown under normal conditions, *AtTps* expression, as analyzed by promoter:*GUS* fusion, is constitutive during seedling development with strong expression in roots and at the root:hypocotyl junction. As seedlings mature, activity becomes undetectable in above ground portions while roots show positive *GUS* staining throughout development. This result is similar to that found in orchid, where *Tps* expression, as analyzed by semi-quantitative PCR, was highest in roots. A model was proposed, for orchid (Chapter 3, Discussion), such that sucrose stimulated trehalose production which in turn induced starch accumulation in leaves. This would mean that sucrose export to roots could be controlled by sucrose-induced expression of trehalose. That *AtTps* expression was constitutive in roots of *Arabidopsis* suggests that sucrose import and trehalose production are balanced. The higher expression of trehalose in roots and cotyledons of seedlings, however, is contrary to the model, as sucrose would be required for growth of seedlings that had not acquired full photosynthetic capabilities. Therefore, if trehalose does promote starch synthesis, genes encoding the enzymes involved in trehalose synthesis would be expected to be down-regulated. In maturing seeds, *AtTps1* expression rises concomitantly with maximum sucrose import and starch

accumulation (Eastmond et al., 2002). It may be that trehalose is necessary to modulate flow of carbon in non-autotrophic tissues and that, since sucrose is the main transport form of carbon, high sucrose levels induce trehalose synthesis. As sucrose levels could be expected to be high in seedlings and during seed fill, expression of *Tps* genes would be high at these stages of growth.

The *AtTps* gene characterized here does not show regulation in response to sucrose or trehalose. In orchid, the *Tps* gene showed up-regulation by sucrose and down-regulation by trehalose. The down-regulation by trehalose is expected, since, if high levels of trehalose do act as a signal, it would be beneficial if trehalose was a feedback inhibitor of its own synthesis. That trehalose does not reduce the synthesis of *AtTps* in *Arabidopsis* is surprising. The concentration of trehalose used in this study is similar to that used by Wingler et al. (2001) who showed that trehalose induced starch synthesis and inhibited root growth, indicating that trehalose, in this concentration, is taken up by the plant. The fact that sucrose had no affect on *AtTps* was also surprising in light of the results from orchid. However, it could be that the *Tps* from orchid and the *AtTps* from *Arabidopsis* are not orthologs. The number of *Tps* homologs in *Arabidopsis* does suggest that different homologs may have different functions.

One response of *AtTps* in *Arabidopsis* was to wounding. This response was rapid (approximately a few minutes) and strong. Analysis of the promoter region of *AtTps* reveals a binding site motif, w-box (TTGACC/T), for the plant transcription factor family WRKY (Eulgem et al., 2000). The WRKY transcription factors have been shown to induce gene expression in response to wounding (Eulgem et al., 1999) and the w-box was present in 80% of genes identified as pathogen inducible (Chen et al., 2002). Though the *AtTps* gene analyzed here did not show a response to interaction with *Thanatephorus pennatus*, it is possible that pathogens may induce expression of *AtTps*. The fungus, *T. pennatus*, is mycorrhizal on orchid. Since *Arabidopsis* plants are not mycorrhizal, it could be that there is no recognition of the fungus. It is also possible that direct contact and penetration is necessary to induce *AtTps*. Though roots of *Arabidopsis* were in direct contact with the fungus, there may not have been any penetration of *Arabidopsis* tissues by the fungus.

The expression of *AtTps* as indicated by *GUS* was high in flowers at a certain developmental stage and in the junction between silique and pedicel. Flower expression was strong in anthers and stigmas of flowers at the stage of development where pollen would be mature and close to being shed. Since pollen is delivered in the dry state, it is possible that *AtTps* expression here is to promote production of trehalose as an osmo-protectant in dry pollen. However, the expression of *AtTps* in stigmas may indicate another role, as stigmatic surfaces are not dry. Trehalose could then be involved in cell-to-cell recognition between pollen and stigma which would be indicative of sugar signaling. Sugars are deposited on the surface of pollen during development (Esau, 1977) and these sugars can cause allergic responses in humans (Di Felice et al., 2001). Sugars may also be involved in gametophytic incompatibility as glycosylations within the recognition site of S-RNases are hypothesized to interact with sugars on the pollen (Mitsukami et al., 1999). Blocking of invertase in tobacco pollen led to male sterility (Goetz et al., 2001) suggesting that sugar import is critical to pollen development. The preceding examples illustrate the importance of sugars to pollen and pollination; however, trehalose is not specifically identified as one of these sugars. Nonetheless, a

role for trehalose in sugar signaling could be critical to pollen development and pollination. Expression of *AtTps* in the silique, pedicel junction may be a function of photo-assimilate partitioning to the developing fruit and again reflecting a role in sugar signaling.

Since multiple genes encode at least two classes of *Tps* homologs in *Arabidopsis*, a general statement regarding interaction between sucrose and *AtTps* cannot be supported. Different genes encoding similar enzymes may be regulated in an organ or tissue specific manner and may respond to different external signals. *AtTps1* shows low levels of expression in all organs with the highest level of expression in seeds (Eastmond et al., 2002). In seeds, maximum expression occurred at the cotyledon stage of development. The *AtTps* gene analyzed here shows highest expression in roots and a strong response to wounding. The presence of two classes of *Tps* also suggests that the gene products may have very different functions. The complementation of yeast *Tps1* by *AtTps1* suggests that it is involved in the production of trehalose. Eastmond et al. (2002) suggest that AtTps1 protein itself could be required for regulation of carbon flow. Since the *AtTps* class II genes cannot complement mutations in either yeast *tps1* or *tps2*, these *AtTps* homologs may not be involved in trehalose synthesis but could be regulators of key metabolic pathways.

The *AtNuBP* promoter driven expression of *GUS* has so far only been observed in pollen. This could suggest a role for AtNuBP in meiosis; however, no *AtNuBP* driven *GUS* was observed in developing siliques where megagametophytes would be undergoing meiosis. In yeast, AtNuBP has been localized to the nucleus (Vitale et al., 1996). It was also shown that amino acid deletions or substitutions of yeast AtNuBP are lethal in haploid spores. This could be due to interference of meiosis or because the mutant phenotype only expressed itself in the haploid. In mouse, a short form of the nucleotide binding protein (lacking the n-terminal cysteine motif) has highest expression in testes, again in conjunction with meiosis (Nakashima et al., 1999). However, the long form of both human and mouse AtNuBP, which is more similar to that in *Arabidopsis* and orchid, has expression in all tissues and organs tested which would preclude a role for AtNuBP in meiosis. It could be suggestive of a role for AtNuBP in mitosis. Further analysis of the *AtNuBP* promoter:*GUS* plants is needed to determine the extent of *AtNuBP* expression in *Arabidopsis*.

The analysis of the *Arabidopsis* homolog of CyPP *Tps* represents the first time a class II trehalose synthesis gene has been characterized. The function of class II *AtTps* genes is believed to be in the second step of trehalose synthesis, the removal of the phosphate from trehalose-6-phosphate. It is also believed that the trehalose synthase domain of these proteins is involved in forming a complex with class I AtTps proteins. Analysis of *AtTps* revealed that it had constitutive expression in roots, and expression in cotyledons. These expression patterns suggest that *AtTps* may have a role in sugar signaling in non-photsynthetic tissues. Expression of *AtTps* was also observed in anthers and on stigmatic surfaces at about the stage of floral development where pollen would be matured and ready for shedding. This could be due to cell-to-cell recognition between stigma and pollen. A strong wound response was also observed in *AtTps* expression, suggesting that sugars may be a component of defense signaling pathways. Expression of *AtNuBP* has only been detected in pollen, which may indicate a role for AtNuBP in meiosis.

Table 4-1. List of components for media used to grow *E. coli* (LB and Terrific Broth) and *Agrobacterium* (2x TY) for use in cloning *AtTps* and *AtNuBP* promoter:GUS fusions and in *in planta transformtion*.

Media	Bacto-Tryptone	Bacto-Yeast extract	Glycerol	K_2HPO_4	KH_2PO_4	NaCl	Bacto-Agar	pH
LB	10 g	5 g	-	-	-	5 g	15 g	7.0
Terrific Broth	12 g	24 g	4 ml	2.31 g	12.54 g	-	-	
2xTY	16 g	10 g	-	-	-	5 g	15 g	7.0