Chapter 3

Analysis of trehalose-6-phosphate synthase/phosphatase and nucleotide binding protein in *Cypripedium parviflorum* var *pubescens*

Introduction

Using amplified fragment length polymorphism (AFLP) differential display, two genes were identified as being differentially expressed in orchid roots in response to a mycorrhizal fungus: nucleotide binding protein (*NuBP*) and trehalose-6-phosphate synthase/phosphatase (*Tps*). Though these genes were confirmed to be differentially expressed, the characterization of their expression is a critical step in understanding their role in mycorrhizal development in orchid.

Trehalose is a common sugar in fungi and was once believed to be a storage sugar (Muller et al., 1999). Its role now seems much more complex. Trehalose accumulates in yeast (*Saccharomyces cerevisiae* Hansen) during the stationary phase of growth and this is concomitant with an increase in activity of trehalose synthesizing genes and a decrease in trehalose degrading genes (Francois et al., 1991; Vuorio et al., 1993). Trehalose has also been shown to be up-regulated in response to stress (Londesborough and Vuorio, 1993). The activity of the trehalose genes has been shown to be regulated by a Ras type GTPase (Uno et al., 1983). In yeast, mutants deficient in trehalose synthesizing genes are unable to grow on glucose (Van Aelst et al., 1993), suggesting that there is cross talk between the glucose and trehalose pathways. Furthermore, it is possible that trehalose acts as a sugar signal and evidence suggests that trehalose-6-phosphate can interact with hexokinase (Blazquez et al., 1993). This would suggest that trehalose plays a role in sugar sensing and signaling.

Until recently, trehalose was thought not to exist in plants. It has been found in the desert plant *Myrothamnus flabellifolia* Welw. (Bianchi et al., 1993) and in species of *Selaginella* (Adams et al., 1990; Koller, 1982) and now in *Arabidopsis* (Vogel et al., 2001). In fact, searches of the Arabidopsis genome reveal 11 copies of the gene (Leyman et al., 2001). Though, trehalose was once thought to be toxic to plants, the prevalence of trehalose synthesizing genes suggests that it may have a role in plant growth and development. Because trehalose is a non-reducing disaccharide, it is believed to have a role in stress protection as it is able to stabilize membranes during heat and water stress (Crowe et al., 1992). Transgenic tobacco exhibiting increased trehalose content show increased drought stress, though their phenotype is often perturbed showing abnormal root development (Romero et al., 1997). Trehalose fed to *Arabidopsis* also leads to stunting of roots and affects carbon partitioning and starch synthesis (Wingler et al., 2000). Again, it is likely that trehalose is acting on sugar signaling pathways. However, trehalose does not seem to affect the activity of hexokinase as it does in yeast (Ian Graham pers. com.; Eastmond et al., 2002). Leyman et al. (2001) suggested that the high number of genes encoding enzymes for trehalose synthesis would indicate a regulatory role for either trehalose or more likely trehalose-6-phosphate.

The protein encoded by *NuBP* from CyPP belongs to a large family of ATPases that includes the bacterial *ParA*, the *NifA* and *ArsA* (Koonin, 1993). The highest degree of homology is with the bacterial *ParA* or partioning ATPases that are believed to have a role in placement of the division machinery during cell division (De Boer et al., 1991). However, the function of homologs in yeast, mouse or human has not been determined (Nakashima et al., 1999; Vitale et al., 1996). The gene is essential in yeast as hemizygous knockouts produce inviable progeny in a 3:1 ratio (Vitale et al., 1996). In Arabidopsis, no work has been done on nucleotide binding protein, though a closely related protein has been shown to play a role in chloroplast division (Osteryoung and McAndrew, 2001). Cloning of the full-length gene was undertaken as well as characterization of expression to understand the role of these genes in CyPP.

Materials and Methods

RACE PCR

Cloning of cDNA ends for both genes was done using the 5' and 3' RACE (Rapid Amplification of cDNA Ends by PCR) kit of Gibco Life Technologies (Carlsbad, California). Briefly, RNA was reverse transcribed using either an oligo dT adaptor primer (3') or a gene specific primer (GSP, 5'). For 3' RACE, cDNA was amplified by PCR with a gene specific upstream primer and a primer specific to the adaptor region used in reverse transcription. PCR was performed at 55°C for 37 cycles. Second round PCR was performed with a nested gene specific primer at 57°C for 35 cycles. For 5' RACE, cDNA was purified from reverse transcription reagents and a 5' poly C tail was added using terminal deoxynucleotide transferase. First round PCR was performed at 55°C with the original gene specific primer and a primer specific to the poly C region of the cDNA. Second and third round PCR was performed at 57°C with nested GSPs and the poly G anchor primer. For a list of primers see Table 3-1.

GeneRacer (Invitrogen) was also used to amplify 5' ends. The terminal phosphate of non-RNAs and truncated RNAs was removed with calf intestinal phosphatase. The remaining RNA was then treated with tobacco acid pyropphosphatase to remove the 5' cap from mRNAs. An RNA oligo was then ligated to the 5' end of mRNAs using T4 RNA ligase. A gene specific primer was used for the RT reaction. For PCR of cDNA, a 5' primer specific to the RNA oligo was used in conjunction with a gene specific primer. Touchdown PCR was used to amplify 5' ends.

RNA Extraction

Extraction of RNA was accomplished using an acid-phenol protocol as described previously. RNA was extracted from roots, rhizomes and above ground portions of seedlings. Extractions were also performed on roots of seedlings grown in the presence of *Thanatephorus pennatus* for 0, 1, 2, 4, 6, 8, 12, 16, and 24 h. Roots of some orchids were grown with *Ceratorhiza goodyera-repentis* (UAMH, Edmonton Alberta, Canada), *Pythium ultimum* Trow. (Dr. Baudoin, Blacksburg, Virginia), or an unidentified ascomycete (isolated from soil under *Kalmia latifolia* L., near Blacksburg, VA). Other

roots were grown in a solution of 50 µM trehalose, 50 µM sucrose, or 50 µM mannitol. All treatments were for 24 h prior to RNA extraction.

RT-PCR Analysis of Gene Expression

Reverse transcription of RNA was accomplished with an oligo 18 dT primer. Template was normalized based on spectrophotometric quantification and gel visualization so that 2 µg of RNA was used per reaction. Primer was added to a concentration of 50 nM and the mixture was heated to 70°C for 15 min. This was placed on ice and a mixture containing 1x buffer, 2.5 mM $MgCl_2$, 10 mM DTT, and 0.4 µM of each dNTP was added. This was heated to 42°C for 1 min and then 1µl of superscript reverse transcriptase was added. This was incubated for 50 min at 42°C and then at 70°C for 15 min. Template was then diluted 1:4 and stored in aliquots at –20°C. For PCR, a 1:100 dilution of the stored template was made and 0.5 µl (0.125 ng) was used per reaction. PCR was performed at 54°C for *NuBP* and at 56°C for *Tps*. PCR using Actin primers was performed to assure normalization of template.

Genomic DNA Isolation

Genomic DNA was isolated using the CTAB method of Doyle and Doyle (1987). Briefly, tissue (0.5 – 1 g) was ground to a fine powder under liquid N_2. 10 mls of warmed (60°C) CTAB extraction buffer was added to the tissue, which was then ground further. This mixture was placed into a 50 ml conical tube and heated to 60°C for 1 h. An equal volume of chloroform:IAA was added and the tubes were vortexed. The sample was then centrifuged at 3000 x g for 20 min. The supernatant was removed to a new tube and an equal volume of chloroform:IAA was added. This was again centrifuged and the supernatant recovered. To this was added 2 volumes of 100% isopropanol and mixed well. This was allowed to sit at –20°C overnight and was then centrifuged at 6000 x g for 30 min. The pellet was washed with 70% ethanol and air-dried. The pellet was resuspended in an appropriate volume of sterilized, distilled water.

DNA Probe Synthesis

Digoxigenin (DIG)-labeled DNA probes were synthesized according to the manufacturer's instructions (PCR DIG Probe Synthesis Kit, Roche Molecular Biochemicals, Indianapolis, Indiana). Template for probe synthesis was the vector containing either the full-length clone of CyPP *NuBP* or a 2000bp partial fragment of CyPP *Tps*. Reactions for DIG labeling (50 µl) consisted of 100 ng of template, 5 µl 10x PCR buffer, 200 µM each dNTP except for dTTP (130 µM) and DIG-11-dUTP (70 µM), and primers (150 nM). Primers used for synthesis of Probes can be found in Table 3-1. PCR conditions were an initial cycle of 94ºC for 5 min followed by 30 cycles of 94ºC for 40 sec, 55ºC for 40 sec and 72ºC for 1 min followed by an elongation step at 72ºC for 7 min. Control reactions were run with the same reaction components and the same cycle parameters without DIG-11-dUTP. To check the integrity of the probe synthesis, control and labeling reactions were run side by side on a 1% agarose gel. The incorporation of

DIG-11-dUTP causes a shift in mobility of the DNA fragment which can be visualized on the gel.

Southern Hybridization

Cypripedium parviflorum genomic DNA was digested at 37°C overnight with *EcoRI*, *HindII*, and *BamHI* restriction enzymes (Promega, Madison, Wisconsin). The resultant digested DNA along with a DIG-labeled DNA marker was subjected to electrophoresis in a 1% agarose gel at 3V/cm for 7 h. The gel was then rinsed in distilled water for 1 h and denatured in 1.5 M NaCl, 0.5 M NaOH for 30 min. The denatured gel was then neutralized in 1.5 M NaCl, 0.5 M TRIS pH 7.0 for 30 min and soaked in transfer buffer (20x SSC) for 30 min. The DNA was transferred to a positively charged nylon membrane (MagnaGraph, Fisher) using standard protocol (Sambrook et al., 1989). The transfer was continued overnight and the DNA was cross-linked to the membrane using the optimal UV scale (UV Cross Linker, Fisher).

The membrane was pre-hybridized at 45°C with 20 ml of UltraHyb hybridization buffer (Ambion, Austin, Texas) for 1 h. DIG-labeled probe was then added (2 µl probe/ 1 ml buffer) and hybridization was carried out overnight at 45°C. Two 15 min low-stringency washes were performed at room temperature in low stringency washing solution (2xSSC, 0.1% SDS). This was followed by a high stringency wash (0.1xSSC, 0.1% SDS) at 45°C.

For detection, the membrane was washed briefly in washing buffer (DIG Wash and Block Buffer Set, Roche) and then blocked for 1 h in blocking solution (Roche). The membrane was then incubated with alkaline phosphatase conjugated anti-DIG antibody (Roche, 1:10,000 dilution) for 30 min. This was followed by two 15 min washes in washing buffer and a brief soak in detection buffer (Roche). The membrane was then incubated with a chemiluminescent substrate (CDP-STAR™, Roche) for 5 min. Signal could be detected on x-ray film after exposures of 30 sec to 2 min.

Results

RACE PCR

The 3' ends of both genes were obtained after 2 rounds of nested PCR using 3' RACE protocol (Fig. 3-1). These were sequenced and scanned for homology with known genes using the BLAST search function of NCBI. For 5' RACE (Fig. 3-2), only a partial fragment of both genes was obtained as revealed by sequencing. Primers were again designed to the 5' end and RACE was re-performed. GeneRacer (Invitrogen) had to be used to generate the 5' end of Tps.

The nucleotide sequence of CyPP *NuBP* (Fig. 3-3) showed highest homology to the corresponding gene from *Oryza sativa* L. whereas the amino acid alignment (Fig. 3-4) showed highest homology to *Arabidopsis thaliana* (Score 543, Evalue e-153, 77% identity, 86% positive). The nucleotide sequence revealed a 5' leader sequence of 172 bases and the initiation codon at position 173. The stop codon is at position 1231 giving an ORF of 1059 base pairs and a protein of 352 amino acids. The 3' UTR is

approximately 120 bp. The deduced amino acid sequence of CyPP NuBP exhibits several motifs consistent with nucleotide binding proteins in other organisms (Fig. 3-3). There is a conserved sequence of CXXXXXXXXXXXXXC XX CXXXXXC near the N terminal site of the protein that is believed to be involved in metal binding. Another feature is the phosphate binding loop, motif A, amino acids 64-81. Finally there are two conserved motifs (motif A' aa 90-101, motif B aa 172-177) that are considered to be signatures of this class of nucleotide binding proteins.

The gene encoding *Tps* cloned from orchid shares high similarity with that of *Oryza sativa* at both the nucleotide and amino acid level. It also shares high homology with the amino acid sequence of *Arabidopsis* and *Solanum tuberosum* L. (Fig. 3-6). The N-terminal half of the protein has high similarity to pfam 00982, glycosyl transferase 20 whereas the carboxy-half of the protein has high similarity to trehalose-6-phosphate phosphatase pfam 02358. Within the phosphatase portion of the gene are two conserved sequences corresponding to phosphatase boxes LDYDGTM and GDDRSH (Fig 3-5.).

Southern Blot and Expression Pattern

In seedlings grown under normal conditions, *Tps* showed highest expression in roots with limited expression in rhizomes and leaves (Fig. 3-7). Expression levels of *NuBP* were highest in leaves with low expression in roots and stems (Fig. 3-7). Southern blot analysis of *Tps* revealed several bands in genomic DNA digested with *EcoRI*, *HindIII* and *BamHI* (not shown). This suggests that *Tps* exists as a multi-copy gene. Southern analysis of *NuBP* revealed only single bands suggesting that this gene exists as a single copy in the genome (not shown).

RT-PCR Analysis of Gene Expression

Actin was amplified to the point where fragments were just visible on an ethidium bromide stained agarose gel visualized with UV light to assure that template in each reaction was basically the same. Adjustments were made to the amount of starting material based on these results so template amount varied from 0.5 µl to 1.4 µl per reaction. This should assure that approximately 0.125 ng of total RNA was used per reaction. The PCR reactions were run so that the treatment with the lowest amount of amplified DNA was just visible on an ethidium bromide stained agarose gel visualized with UV light. This made visual quantification easier.

Amplification of *NuBP* revealed that its expression increased over time with the greatest increase from 8-24 h (Fig. 3-8). Contrary to the initial results, *Tps* showed increased expression, with undetectable expression in untreated plants (Fig. 3-8). The level of *Tps* expression as analyzed by PCR varies after incubation with the highest level at 12 h. It is important to note that both genes show a background level of expression but that interaction with the fungus does alter their expression. The results were confirmed by repeating the experiment (data not shown). While there were some differences, the overall patterns were the same.

When plants were grown in the presence of another fungus (*Ceratorhiza goodyera-repentis*) the results were different (Fig. 3-9). Up-regulation of *NuBP* was observed in plants grown with the fungus treatment whereas *Tps* showed down-

regulation. Plants incubated with either *Pythium ultimum* or an unidentified ascomycete did not show the same results as the mycorrhizal fungi (Fig. 3-10). Whereas *Tps* did show slight down-regulation there was no change in expression in *NuBP*. When plants were grown in the presence of 50μM trehalose, *Tps* showed down-regulation, whereas *NuBP* showed up-regulation. When plants were incubated with 50 μM sucrose (a sugar that is somewhat similar to trehalose) and 50 μM mannitol *Tps* showed slight up-regulation. There was slight up-regulation of *NuBP* when plants were incubated with sucrose and mannitol though not to the extent seen when roots were incubated with trehalose. Plants grown on oatmeal agar for 24 h showed similar expression to plants grown in perlite.

Discussion

Trehalose is a disaccharide consisting of two glucose moieties linked in an alpha 1, 1 configuration. It was long believed to be absent from plants and was thought to be toxic (this is true for some plant species). Free trehalose had, until recently, only been found in the desert plant *Myrothamnus flabellifolia* (Bianchi et al., 1993) and in species of *Selaginella* (Adams et al., 1990; Koller, 1982) though the enzymes for synthesis of trehalose have been found in other plant species. Trehalose has now been detected in *Arabidopsis* (Vogel et al., 2001) indicating that the genes encoding trehalose synthesis are functional. The low, almost undetectable levels of trehalose in most plants raises questions as to its role in plant growth and development. However, speculations about its function range from desiccation tolerance, to a signaling molecule, to a role in plant interactions with other organisms (Muller et al., 1999). An *Arabidopsis* mutant deficient in *Tps1* has been identified and is embryo lethal suggesting that at least one member of the *Tps* family is essential for development in *Arabidopsis* (Eastmond et al., 2002).

Three genes for trehalose-6-phosphate synthase/phosphatase (*Tps*) have been cloned from *Arabidopsis* and two genomic clones are known in rice. From the eleven *Tps* genes identified in the *Arabidopsis* genome, two major classes can be distinguished based on their homology to each other and to the yeast *Tps* genes (Leyman et al., 2001). The first class has high homology to yeast *Tps1*, which lacks a phosphatase domain. The second class has a large C-terminal extension that has a high degree of homology with the yeast trehalose phosphate phosphatase (Leyman et al., 2001). Leyman et al. (2001) speculated that the *Tps1* class is actively involved with the first step in trehalose synthesis, the formation of trehalose-6-P from UDP-glucose and glucose-6-P, while the *Tps2* class is involved in removal of the phosphate to produce trehalose. Further evidence for this can be seen in the functional complementation of yeast *Tps1* mutants. *AtTps1* is able to complement the mutation whereas *AtTpsA* and *AtTpsC* (both class two Tps genes) cannot complement the mutation (Vogel et al., 2001). However, neither *AtTpsA* nor *AtTpsB* can complement mutations in yeast *Tps2*, the phosphatase containing gene. The function of the class II *Tps* genes is complicated by the fact that specific trehalose-6-phosphatase genes exist in *Arabidopsis* that are able to complement the yeast *tps2* mutant (Vogel et al., 1998). However, these genes have low overall homology with other trehalose-6-phosphate phosphatases and Leyman et al., (2001) speculated that they are non-specific sugar phosphatases. The gene cloned from orchid roots grown without a mycorrhizal fungus shows high homology to that of *Oryza sativa* and *Arabidopsis thaliana* and specifically with the *Tps2* class.

As mentioned, little is known about the function of trehalose in plants. Since the trehalose found in *Selaginella* was found in desiccated plants, one function is that of desiccation tolerance. However, attempts to create transgenic plants over-expressing trehalose synthesizing genes have led to plants with aberrant phenotypes. In *Nicotiana*, for example, perturbed root growth and altered leaf morphology accompanied the increase in drought tolerance (Romero et al., 1997). Furthermore, blocking trehalase activity while feeding *Arabidopsis* plants trehalose led to dwarf plants with altered root architecture (Wingler et al., 2001). The same study also found that increased trehalose altered root:shoot carbon partitioning and led to increased starch synthesis through the activity of ADP-glucose pyrophosphorylase. The suggestion was made that trehalose acts as a sugar signal directing the translocation and storage of photosynthate. The expression of orchid *Tps* seems to be highest in roots with some expression in leaves and rhizomes. Coupled with the above data, this would suggest that trehalose has a role in carbon metabolism in non-photosynthesizing tissues. It is possible that trehalose produced in roots has a role in normal carbohydrate partitioning to this sink tissue. A simple model can be proposed (Fig. 3-11) in which sucrose is an activator of trehalose-6-phospate synthase/phosphatase. When enough sucrose is available to roots, trehalose is produced and is translocated to the shoots where it stimulates starch accumulation (the mechanism of trehalose transport is unknown and it is possible that trehalose acts indirectly in shoots via a second messenger). As sucrose is converted to starch less is available for export to the roots where sucrose levels would drop. Trehalose levels would lower due to a lack of stimulation of *Tps*. The lack of trehalose exported to shoots would lower starch synthesis, freeing more sucrose for export to the roots. This would explain why increasing external trehalose leads to starch synthesis and slowed root growth. The data that shows that sucrose increases *Tps* expression adds support to this model. Furthermore, down-regulation of *Tps* by a fungal symbiont would lead to increased sucrose partitioning to roots, which would provide the symbiont with fixed carbon

Differential display identified *Tps* as being down-regulated by the fungus. This was confirmed by RT-PCR and in a later screening of plants for changes in expression due to various factors. A time-course study of interaction of the orchid with the fungus showed that *Tps* was up-regulated in response to the fungus (Fig. 3-8). The plants used for this analysis were newly removed from dormancy whereas in the other studies, plants had completed their seasonal growth and, though actively photosynthesizing, may have been preparing for the next dormant period. In terms of the aforementioned model, these data are more difficult to interpret. However, it may relate to seasonal changes in mycorrhizal status of the orchid. In spring, the orchid is breaking dormancy and is reliant on stored carbon for early growth. It may also utilize the fungus as a carbon source. Since carbon is being transported to shoot growing points, it may be necessary to prevent sucrose export to the roots. An increase in trehalose would accomplish this according to the above model. Furthermore, with the addition of carbon from an external source, it is less necessary for carbon to be transported to the roots. When growth has ceased for the season, the plant is not as dependent on fixed carbon except for storage. In this case, the plant may provide the fungal partner with fixed carbon. The down-regulation of trehalose synthesis would provide this carbon to the roots according to the model. It is plain to see, however, that the regulation of trehalose by interaction of the orchid with the fungus is complex and goes beyond carbon partitioning.

Expression analysis of *Tps* revealed that incubation with two different mycorrhizal fungi reduced expression. This suggests that the interaction between *Cypripedium* and orchid mycorrhiza is non-specific. The two species of fungi used in this study were isolated from orchids other than *Cypripedium*, and confirmation of mycorrhizal formation in *Cypripedium* has not been done. However, incubation of CyPP with two non-mycorrhizal fungi did not lead to a change in expression of *Tps*. This indicates that the response is due to mycorrhiza and not to fungi in general.

The cloning of *NuBP* represents the first time this gene has been cloned from a plant species other than *Arabidopsis*. The gene is known in rice, but only through genomic BAC clones. *NuBP* from CyPP shows striking similarity at the amino acid level to that from *Arabidopsis* and even has high degree of homology with nucleotide binding proteins from yeast, mouse and human. The motifs characteristic of this gene are present and share greater than 80% identity with *Arabidopsis* and the proteins share 77% identity overall. This suggests that the gene is highly conserved and has a critical function within the plant. Further evidence for a critical role in plant growth and development can be found in the fact that CyPP *NuBP* appears to exist as a single copy gene as is the case in *Arabidopsis*.

In bacteria, MinD, the homolog of the plant nucleotide biding protein, is associated with chromosome partitioning and cell division (de Boer, 1991). Bacterial MinD acts in concert with other proteins to promote formation of the septum at the proper site for cell division. Disruption of MinD results in the formation of small cells that cannot divide (Colletti et al., 2000). This is believed to be due to the fact that without MinD, the division apparatus develops at sites other than that required for normal cell division. Colletti et al., (2001) has found a MinD homolog in *Arabidopsis* (*AtMinD1*) that is necessary to division of the chloroplast and mutations of which result in aberrant chloroplast development.

A homolog of *MinD*, *AtMinD1*, was identified in *Arabidopsis* by screening the *Arabidopsis* genome with an amino acid sequence of *Chlorella vulgaris* Beijerinck *MinD* using TBLASTN (Colletti et al., 2000). It was found to share 65% amino acid identity with the *Chlorella vulgara MinD*, 50% with other algae and 40% with non-photosynthetic bacteria. The ATP binding site (motif A), motif A' and motif B are all largely identical between AtMinD1 and the other MinD proteins. However, an alignment of *AtMinD1* with CyPP *NuBP* reveals that, while there is a 100% identity with the ATP binding site, there are differences in both motif A' and motif B. Overall, *AtMinD1* and CyPP *NuBP* share only 28% homology. Furthermore, *AtMinD1* has an N-terminal extension that is believed to be a chloroplastic targeting sequence (Colletti et al., 2000). Orchid NuBP lacks a targeting sequence and prediction of localization using ProSite (http://www.expasy.ch/prosite) suggests that NuBP is cytosolic. The low level of identity between *NuBP* and *AtMinD1* suggests that while the CyPP *NuBP* is related to *AtMinD1*, it is not the same and its function may differ. That is, it is probably not involved in chloroplast division.

The *Arabidopsis* protein with which CyPP *NuBP* shares 77% identity is also significantly different from *AtMinD1* suggesting that there is a second class of nucleotide binding proteins in plants that has a different function from that ascribed to *AtMinD1*. These proteins both share significant homology with nucleotide binding proteins from yeast, mouse and human. Since these organisms lack chloroplasts it strengthens the

argument that CyPP NuBP and related nucleotide binding proteins are not involved in chloroplast division. It is tempting to speculate that, with their high degree of similarity to bacterial MinD and ParA type proteins, CyPP NuBP and related nucleotide binding proteins are involved in mitochondrial division. However, to date, no one has identified proteins associated with mitochondrial division.

A role for CyPP *NuBP* in mitochondrial division could have significance for mycorrhizal interactions. Since CyPP *NuBP* showed an increase in expression in response to the fungus, it is possible that interaction with the fungus could be stimulating mitochondrial division. The mitochondrion is not just a site of TCA, but a dynamic organ that has a role in diverse metabolic functions due to the interaction of TCA intermediates in various biosynthetic reactions. Amino acid pools, especially those dependent on aspartate and glutamate, are derived from TCA cycle intermediates. Isoprenoids are synthesized from acetyl CoA, which is believed to be generated from citrate derived from the mitochondrion. With the formation of a symbiosis there could be an increased need for amino acids as gene expression leads to increased protein levels. Furthermore, the need for carbon in a form required by the fungal partner may be filled by the mitochondrion. It is also possible that synthesis of defense compounds takes place and this would be provided, in part, from isoprenoids. Finally, an increase in energy consumption in the form of ATP is probably concomitant with the formation of the symbiosis and the mitochondrion would be required to provide additional ATP.

Another possibility for the function of *NuBP* could be in regulating events within the nucleus during cell division. Though they are distantly related, the ParA class of nucleotide binding proteins are involved with chromosome partitioning during bacterial cell division. Vitale et al., (1996) showed that the yeast *Nbp35* was localized to the nucleus suggesting that it could be related to chromosome replication. This gene is necessary in yeast as knockout mutants produce inviable progeny in a 1:1 ratio. In human and mouse, *NuBP* is expressed in various organs, tissues and throughout development (Nakashima et al., 1999), again, suggesting a crucial role. If *NuBP* is involved with chromosome replication or division, its role in the mycorrhizal association could be more difficult to interpret. However, in some orchids, endoreduplication of DNA has been seen to occur upon infection with a mycorrhizal fungus (Arditti, 1992, Raghaven and Goh, 1994).

The addition of trehalose to orchid plants increased the expression of *NuBP*. If trehalose does alter the flow of carbon to the roots and if *NuBP* is involved in mitochondrial division then the increase in NuBP expression may be a response to low carbon. With carbon starvation, it may be necessary to divert carbon from other sources. Since the mitochondrion is such a dynamic organelle, which partakes in many anabolic and catabolic pathways, it would be essential for regulating carbon flow. Furthermore, if during new growth the fungus is providing carbon, the mitochondrion would be vital in converting it to useable forms and directing it to other uses. Alternatively, trehalose could be a signal from the orchid that leads to endoreduplication of DNA. If *NuBP* is involved in replication of DNA, its expression would be increased with higher amounts of trehalose from external sources.

The expression of CyPP *Tps* and *NuBP* was analyzed using semi-quantitative PCR. Unique roles can be proposed for their function based on their pattern of expression and response to stimuli. *Tps* is up-regulated by sucrose, but down-regulated

by trehalose and interaction with mycorrhizal fungi. The expression of *Tps* is highest in roots and moderate to low in rhizomes and leaves. It is suggested that the potential product of *Tps*, trehalose, is a signal that directs carbohydrate partitioning in response to internal needs of the plant as well as to external stimuli such as interaction with the mycorrhizal fungus. *NuBP* is up-regulated by fungi and trehalose. Though similar to AtMinD1, a nucleotide binding protein from *Arabidopsis* that is important to chloroplast division, this function cannot be assigned to CyPP NuBP based on computer alignments and analysis of the deduced amino acid sequence. It is possible that the product of *NuBP* is involved in mitochondrial division and that NuBP indirectly promotes an increase in metabolism, through increased number of mitochondria, to respond to changes in growth and to interactions with other organisms.