## Summary

A differential display technique was used to identify changes in gene expression in a native orchid in response to a mycorrhizal fungus. An initial attempt at differential display was made using a fluorescence-based technique with seeds of *Goodyera pubescens*. Samples of RNA from dry seeds and imbibed seeds were reverse transcribed with an oligo18 dT VV primer and samples were amplified by PCR using a fluorescent labeled oligo dT V primer and random upstream primers. Amplification was detectable, though the detection limit for fluorescent identification of individual bands proved too low. An amplified fragment length polymorphism (AFLP) base method was used with $\alpha$ $P^{33}$ dATP as the label. Reverse transcribed RNA from roots of *Cypripedium parviflorum* var. *pubescens* (CyPP) grown in the presence or absence of a mycorrhizal fungus, *Thanatephorus pennatus*, was used in AFLP-DD PCR analysis. Clones selected as being differentially expressed were subjected to BLAST for identification. Of approximately 5000 fragments, 44 were selected as differentially expressed. Of those, only 15 sequences were obtained. Most of these encoded ribosomal genes. Two represented genes believed to be regulated by the mycorrhizal interaction: trehalose-6-phosphate synthase/phosphatase (*Tps*), which showed down-regulation and nucleotide binding protein (*NuBP*), which showed up-regulation. The differential expression of these genes was confirmed using semi-quantitative PCR.

The identification of ribosomal genes was unexpected but may reflect a change in metabolism. These genes are involved in protein synthesis and thus may relate to changes in protein production in response to the fungus. Others have reported that ribosomal genes can be identified by differential display and likely account for false positives inherent in the procedure.

To characterize genes identified as being differentially displayed in roots of CyPP grown in the presence or absence of a mycorrhizal fungus, the full sequence of each gene was determined and the expression of each gene was analyzed via RT-PCR analysis of gene expression. To clone the full length of each gene RACE PCR was performed. This provided the 5' and 3' ends of each gene. The whole gene was then cloned and sequenced by PCR. The 600 bases presumed to be the most 5' end of trehalose-6-phosphate synthase/phosphatase have still not been cloned. The available sequence of *Tps* is approximately 2100 bp encoding a protein of 667 amino acids. The protein shows high homology to similar genes in rice, potato and *Arabidopsis*. Sequence alignment with *Arabidopsis Tps* genes suggests that it is a class II *Tps* gene containing a c-terminal extension with motifs characteristic of trehalose-6-phosphate phosphatases. *NuBP* is approximately 1300 bp encoding a protein of 353 amino acids. The nucleotide sequence shows high homology to a rice *NuBP* whereas the amino acid sequence shows high homology to nucleotide binding proteins from *Arabidopsis*, yeast, mouse and human. Sequence alignments reveal several motifs characteristic of this type of nucleotide binding protein.

Expression of CyPP *Tps*, as determined by semi-quantitative PCR, was highest in roots with low to moderate expression in leaves and rhizomes. *Tps* showed up-regulation in newly growing seedlings, with induction one hour after incubation with the fungus. Incubation of CyPP with another endophyte, *Ceratorhiza goodyera-repentis*, led to down

regulation of *Tps* as had been found previously. Incubation of CyPP with a broad spectrum plant pathogen, *Pythium ultimum*, and a non-mycorrhizal fungus did not influence *Tps*. The response of *Tps* to the various fungi suggests that the mycorrhizal fungus is most likely to induce changes in *Tps* expression. Trehalose led to reduced expression of Tps whereas sucrose increased it. The affect of sucrose on *Tps* was not surprising as Tps or its products trehalose and trehalose-6-phosphate have been identified as regulators of sugar metabolism. This could have significant implications for mycorrhizal association as carbon flow between host and fungus is critical to formation of the symbiosis. It may be that fungal sugars interact with normal plant sugar signaling and increase flow of carbon to the roots where it would be more available to the fungus.

For *NuBP*, expression increased over time with maximal increases seen from 8-24 h of incubation of CyPP with the fungus. *Ceratorhiza goodyera-repentis* also led to increased expression of *NuBP* whereas *Pythium* and a non-mycorrhizal fungus did not. Sucrose did not affect *NuBP*, but trehalose induced expression. The possibility exists for NuBP to be involved in mitochondrial division, which would be a crucial juncture for carbon flow in the plant in response to a mycorrhizal fungus.

To characterize *Tps* and *NuBP* further and to confirm the results from analysis in orchid, promoter:*GUS* fusions were created in *Arabidopsis*. Analysis of gene function in orchid using genetic techniques (knockouts, mutation, promoter:reporter gene constructs) is not easy due to the large genome size, long reproductive span and difficulties in transformation. Therefore, *Arabidopsis* was used since homologs of both genes identified by differential display are present in *Arabidopsis*. Though orchids and Arabidopsis are quite different, the use of functional genetics in a heterologous system can be informative.

Promoter:*GUS* fusions for both *Tps* and *NuBP* were created in pBI 121. The promoter of each gene was cloned from the homolog in *Arabidopsis* that showed the highest similarity in a BLAST search. The CaMV 35S promoter was cut from pBI 121 and replaced with the *Arabidopsis* promoter. Promoter:*GUS* fusions were transformed into *Arabidopsis* via *Agrobacterium*. Seed was harvested from transformed plants and germinated on flats of commercial potting mix. Seedlings were sprayed with kanamycin and susceptible seedlings removed. Plants showing resistance to kanamycin were screened for *GUS*. PCR was used to confirm presence of the transgene.

Transformation of *Arabidopsis* with *NuBP* promoter:*GUS* produced plants showing *GUS* expression in developing pollen. This finding would suggest that NuBP is not involved in mitochondrial division, as it cannot be expected that mitochondrial division would be greater in pollen than in other organs. A role can be proposed for the protein encoded by *NuBP* in meiotic division, though *NuBP* driven *GUS* was not identified during mega-gametophyte development. It remains to be seen if *NuBP* driven *GUS* is present in roots.

In *Arabidopsis* plants transformed with *AtTps* promoter:*GUS* fusions, three lines showed *GUS* expression though only two showed positive for the insert using PCR. Promoter driven *GUS* was observable in seedlings until the time when the first leaves appeared. Expression was concentrated in roots with strong expression in the junction between the root and the hypocotyl. Expression was light but remained in roots throughout development. Activity of *GUS* could also be seen in flowers about the time of pollen maturity. Activity was concentrated in anthers and on the stigmatic surface.

There was also activity in the junction between the silique and the pedicel. Activity of *GUS*, as regulated by the *Tps* promoter, was also strongly up-expressed by wounding. Expression of *Tps* regulated *GUS* in roots, seedlings and the silique:pedicel junction could be ascribed to sugar metabolism. In roots and seedlings, photosynthesis is either absent or not at peak efficiency. Sucrose import into organs at these times is crucial to growth and expression of *Tps* may be related to the high levels of sucrose import into these sink tissues. Expression of *Tps* in the pedicel:silique junction could also reflect a role for the Tps protein in sugar metabolism since sucrose would be shuttled to developing siliques for storage in developing seeds. The wound response of *Tps* driven *GUS* may reflect a role for sugar metabolism in defense related signaling pathways.

Two genes identified as differentially expressed in roots of an orchid (*Cypripedium parviflorum* var. *pubescens*) grown in the presence of a mycorrhizal fungus (*Thanatephorus pennatus*) were characterized in orchid and in *Arabidopsis*. The gene *NuBP*, which encodes a protein related to the bacterial cell division factor MinD, showed up-regulation in response to the fungus. Functional characterization undertaken in this work suggests that *NuBP* is regulated by trehalose and by mycorrhizal fungi. In *Arabidopsis*, *NuBP* is expressed in developing pollen. It was thought that *NuBP* could be involved in mitochondrial division, though that now seems unlikely. Further characterization of this gene should reveal its function in *Arabidopsis* and in orchid.

The gene (*Tps*) encoding an enzyme involved in synthesis of trehalose-6-phosphate and trehalose was down-regulated by the interaction. Further analyses support the hypothesis that the gene products of *Tps* or the compounds synthesized by these gene products are involved in sugar signaling. This may have significant implications for mycorrhizal formation in orchids. Sugar flow between orchid and fungus is probably tightly regulated and could be crucial to establishment and maintenance of the symbiosis. Trehalose has been studied in terms of its effect on orchid seed germination with limited success. It may be that trehalose-6-phosphate is more potent an activator or inhibitor of sugar response pathways, but sugar phosphates are not easily taken up by plants. The work presented here has identified two genes that show differential regulation in *Cypripedium parviflorum* var. *pubescens* in response to *Thanatephorus pennatus*. The work has also provided characterization of both these genes in orchid and in *Arabidopsis*.