Chapter 2
The use of differential display to identify changes in gene expression brought about by the interaction of a mycorrhizal fungus with a North American terrestrial orchid

Introduction

With the prevalence of mycorrhizal symbiosis in the plant kingdom it is essential to understand the mechanisms underlying the formation and maintenance of the symbiosis. Much work has been done in both the vesicular arbuscular mycorrhiza and ectomycorrhizal forms of this symbiosis, but the less commonly represented forms of mycorrhiza have not been studied at the molecular level. Though the orchidoid and monotropoid mycorrhizas are not common in the plant kingdom they are unique, sharing similarities with both ecto- and endo-mycorrhizae. Characterization of molecular interactions in both host and fungus of these interactions will reveal further similarities with the more familiar mycorrhizae. Furthermore, it will allow researchers to determine the evolutionary changes that allowed these types of mycorrhiza to arise. Also, since these types of mycorrhiza tend to favor plant growth over that of fungus it can be said that these types of mycorrhiza are in essence reverse parasitism. That is, the plant is utilizing the fungus as a source of energy. This would suggest that the plants involved in this type of symbiosis are capable of overcoming fungal defense and suppressing fungal virulence, both characteristics that could be exploited to improve plant disease resistance.

As mentioned, the orchidoid mycorrhizal symbiosis is unique. All orchids are fully mycotrophic at seed germination and some species are mycotrophic throughout their lives. Orchids display a wide range of dependence on the symbiont after germination which makes them an excellent system to study plant mycorrhizal relations. Also, orchids make excellent horticultural subjects and some (i.e., temperate terrestrial species) are limited in production due to failure to establish the mycorrhizal symbiosis under nursery conditions. Study of this interaction may lead to better production methods of temperate terrestrial orchids.

To investigate the interaction between orchids and fungi at the molecular level two approaches can be taken. One of these is a directed approach, searching for homologs to genes identified in other mycorrhizal symbioses. The other is a non-targeted approach to look for any genes that show regulation in response to the fungus. Because the presence of a gene in one type of mycorrhizal symbiosis does not guarantee its action in another type, a non-targeted approach was chosen for this study. Furthermore, the non-targeted approach has the benefit of revealing novel genes involved in symbiosis.

Differential display is a powerful technique that has been used to identify changes in gene expression that lead to certain phenotypes or that arise in response to some external factor. Liang and Pardee (1992) first introduced differential display in 1992 and based their methodology on RAPDs. Amplification of cDNA is done with a short random primer and a labeled oligo dT primer. A 2 or 3 bp overhang at the 3' end of the oligo dT primer limited the number of fragments generated by a primer pair. Labeling of the oligo dT primer meant that only fragments amplified by both primers could be visualized on a gel. The primary benefit of differential display is that a relatively wide

subset of genes can be screened for regulation in a short amount of time. Furthermore, there is not a need for large quantities of RNA.

The use of differential display has been widespread and has revealed gene changes in *Arabidopsis thaliana* (L.) Britton (Kreps et al., 2000), tobacco (Kimura et al., 2001), mosquito (Morlais and Severson, 2001), and humans (Doug et al., 200; Klumar et al., 2001). It has also been used to identify changes in plant gene expression in response to fungi (Collinge and Boller, 2001), mollicute (Jagoueix-Evillard et al., 2001), nematodes (Jones and Harrower, 1998) and bacteria (Swiderski et al., 2000). It has even been used to identify genes involved in arbuscular mycorrhiza in pea (Martin-Laurent et al., 1997). Though there are difficulties associated with differential display (Appel et al., 1999; Debouck, 1995; Nagel et al., 2001), there have been improvements made to the technique to reduce the incidence of false positives (Bachem et al., 1998; Habu et al., 1997; Nagel et al., 2001). In this study, differential display will be used to reveal orchid genes that show regulation in response to interaction with a mycorrhizal fungus.

Materials and Methods

Plant and Fungal Material

Seeds of *Goodyera pubescens* (Willd.) R. Br. used to test the fluorescence differential display were collected from wild populations in Montgomery County, VA and stored at 4°C. Roots and stems of *Epidendrum radicans* Pav. used to test the AFLP differential display protocols were obtained from the Virginia Tech horticulture greenhouse. Seedlings of *Cypripedium parviflorum* var. *pubescens* (Willd.) Knight were purchased from Spangle Creek Labs (Bovey, Minnesota). Seedlings were maintained at 4°C in sealed plastic bags (Zip-Loc) with about 5 mls of water until use. To grow seedlings, they were removed from the refrigerator, potted in 7.5 cm. pots of perlite, and watered as needed with de-ionized water. At every other watering plants were watered with a dilute fertilizer. Cultures of *Thanatephorus pennatus* Currah were purchased from the University of Alberta Mycology Herbarium (UAMH, Edmonton, Alberta, Canada). Cultures were kept on oatmeal agar (see below) and sub-cultured every 6 months. For use, 0.1 cm squares of culture were used to innoculate a 150 mm plate of oatmeal agar and grown for two weeks prior to use. Oatmeal agar (modified from Warcup, 1973) (500 ml): 5 g ground oatmeal, 0.5 g maltose, 0.5 g 2(N-morphilino) ethanesulfonic acid (MES), 0.05 g myo-inositol, 0.5 g yeast extract, 7.5 g Bacto-agar pH=5.0.

RNA Extraction

A hexadecyltrimethylammonium bromide (CTAB) method (Knapp and Chandlee, 1996) was used to extract RNA from seeds of *Goodyera pubescens* imbibed in water or on oatmeal agar. Basically, 50-100 mg imbibed seeds were ground in 1 ml warm (65°C) 3% CTAB buffer (3% w/v CTAB, 1.42 M NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Tris-HCl pH=8.0, 2% w/v polyvinylpyrrollidine, 5 mM ascorbic acid) to which was added 10 µl 2-mercaptoethanol. After the extract was ground, it was heated at 65°C for 20 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed. The sample was then spun in a microfuge (Eppendorf 5415) at 10,000 rpm for 10 min. The supernatant was removed and one-fifth volume of 5% CTAB (5%

CTAB, 0.7 M NaCl) was added and the sample was reheated for 20 min. This was re-extracted with chloroform:isoamyl and the supernatant was removed to a new tube. To precipitate the RNA, one-tenth volume of 7.5 M NH_4OAc and 2 volumes of 100% ethanol were added and the tubes were placed at –20°C for 1 h to overnight. The sample was then centrifuged at 14,000 rpm for 10 min. The pellet was washed in 70% ethanol and resuspended in an appropriate volume of Diethyl Pyrocarbonate (DEPC) treated water.

For extraction of RNA from roots and stems of *Epidendrum* and from roots, leaves and rhizomes of *Cypripedium*, a standard phenol extraction was used (Mann Lab, Columbia University, New York, New York, pers. com.). Plant tissue was ground under liquid nitrogen and placed in an appropriate volume of extraction buffer. The extraction buffer for 1-2 g of tissue consisted of 10 ml grinding buffer (180 mM Tris, 90 mM LiCl, 4.5 mM EDTA, 1% SDS pH=8.2), 10 ml phenol:chloroform pH=4.7, 0.9 ml 2 M NaOAc pH=4.5, and 10 µl mercaptoethanol. The sample was then vortexed and homogenized with a polytron homogenizer. This was done 3 times for 1 min each time with a 1 min rest between each step. The sample was kept on ice for the entire grinding period. The sample was then centrifuged at an RCF of 5000 x g for 20 min. The supernatant was removed to a new tube and an equal volume of phenol:chloroform:isoamyl pH=8.0 was added. This was centrifuged at 5000 x g for 20 min and the supernatant was recovered to a new tube. An equal volume of chloroform was added and the sample was centrifuged at 500 x g for 20 min. The supernatant was recovered and 2 volumes of 8 M LiCl were added. The sample was then stored at –20°C overnight. RNA was precipitated by centrifugation for 30 min at 10,000 x g. The pellet was washed with 70% ethanol and air-dried. It was then resuspended in an appropriate volume of DEPC treated water with 0.1mM EDTA.

After extraction of RNA, samples were brought to a total volume of 25 µl with DEPC water. To this was added 2.5 µl of 1 M Tris pH=7.0, 20 µl 25 mM $MgCl_2$, 0.5 µl RNasin, 2 µl of RNase free DNase. The final concentrations were 100 mM Tris, 10 mM $MgCl_2$, 20 units of RNase inhibitor, and 20 units of RNase free DNAse. The sample was heated at 37ºC for 30 min. After DNAse treatment the sample was re-extracted with an equal volume of phenol:choloroform:isoamyl (25:24:1), precipitated with one tenth volume 7.5 M NH_4OAc and 2 volumes of 100% ethanol, and washed in 70% ethanol. The pellet was resuspended in 20 µl DEPC treated water with 0.1 mM EDTA. RNA was quantified via a spectrophotometer. An aliquot of RNA was run on a 1% agarose gel in formaldehyde TBE buffer to check integrity.

Fluorescence Differential Display

The methodology in a kit from Display Systems Biotech (now Azign Bioscience A/S, Copenhagen, Denmark) was followed to perform differential display. RNA from seeds of *Goodyera pubescens* imbibed in water or on oatmeal agar for 12-24 h was used to estimate the practicality of the differential display protocol. For differential display analysis, 300 ng RNA was reverse-transcribed with an oligo 18 dT VV and 27 units of MMLV reverse transcriptase. RNA and the primer was heated to 70ºC for 10 min and then placed immediately on ice. All other reagents as a master mix were then added and the reaction was incubated at 42°C for 1 h. In a 30 µl reaction the concentrations of

reagents were as follows: 1x RT buffer, 125 μM dNTP, 2.5 μM primer. RNAsin was included at 20 units per reaction.

Reverse transcribed RNA or cDNA was used directly in the differential display protocol. The kit purchased from Display Systems Biotech had three fluorescently labeled downstream primers: Cy5 or FAM –5'G T15 GG, GC or GA. For analysis of differential expression, one labeled downstream primer was used in combination with eight upstream primers (Table 2-1), each pair in a different reaction tube. Reaction components were 1 μl of cDNA (10 ng), 1x reaction buffer, 1.5 mM $MgCl_2$, 50 μM dNTPs, 2.5 μM downstream primer, 0.5 μM random primer and 1 unit of Taq DNA polymerase. The Taq supplied by the manufacturer was used, as it is specific for display profiling. PCR was carried out on a Stratagene Robocycler (La Jolla, California) with heated lid. Reaction conditions were 1 cycle of 3 min at 94°C, 5 min at 40°C, and 5 min at 72°C. This was followed by 38 cycles of 30 sec at 94°C, 1 min at 40°C and 1 min at 72°C. A final extension cycle of 5 min at 72°C was also used. This protocol was changed to initial melt at 94°C for 3 min followed by 38 cycles of 94°C for 1 min, 40°C for 1 min and 72°C for 1 min. The final extension was kept the same.

AFLP cDNA Differential Display

To analyze changes in gene expression using amplified fragment length polymorphism (AFLP) cDNA differential display, a kit was used as supplied by Display Systems Biotech. Detection of bands was accomplished with αP^{33} ATP. The use of αP^{33} ATP is better for resolution of bands due to its low energy emission of radiation. Roots of *Cypripedium parviflorum* var. *pubescens* grown in the presence and absence of the fungus were used for RNA extractions. For treatment with fungus, seedlings were transferred to plates of oatmeal agar overgrown with *Thanatephorus pennatus*. Water was added to prevent desiccation of roots and fungus and the plate was swirled at 23°C. After 12 h, orchid seedlings were removed from the fungus and the roots were placed in liquid nitrogen. Roots of untreated seedlings were harvested and placed in liquid nitrogen. RNA extractions were performed immediately following harvesting of roots. Approximately 12 plants were used per extraction. For AFLP based differential display, RNA was reverse transcribed with an oligo 18 dTV primer and MMLV reverse transcriptase. Reverse transcription was carried out at 42°C for 2 h with no heating of cDNA. Final concentrations were 1 μg RNA, 0.75 μM primer, 1x buffer, 1 mM dNTPs and 100 units of reverse transcriptase. The cDNA was then nicked with RNase H and second strand synthesis was performed with DNA polymerase I. This was performed at 16°C for 2 h. Reaction mixture was 75 μl and consisted of 25 μl first strand reaction, an additional 0.25 mM dNTP, 1x buffer, 0.8 units RNase H, 10 units DNA polymerase I. After termination of the reaction for 10 min at 75°C, the double stranded cDNA was brought to a total volume of 200 μl with DEPC water, extracted with an equal volume of phenol:chloroform, precipitated with ethanol and resuspended in 20 μl DEPC treated water. Half the sample was then digested with *TaqI* restriction enzyme. After digestion two adaptors were ligated to the cut ends of the cDNA. One adaptor has an extension protection group. The resultant template for DD-PCR was then diluted 1:4. For PCR, 1 μl of template was added to PCR cocktail containing 1x PCR buffer, 0.03 mM dNTPs, 0.2 μM downstream or 0-extension primer, 0.2 μM probe or gene specific primer and 1μCi αP^{33} dATP. The 0-extension primer anneals to the adaptor with the extension

protection group and cannot bind during first round synthesis. This means that if 0-extension primers are in opposing orientations no amplification can occur. The other primer has a 3 bp overhang in to the cDNA fragment. This specifies the primer for a specific subset or expression window of the expressed genes. There are 64 of these expression windows and 64 probe primers. PCR is carried out for 35 cycles at 94°C melting, 55°C annealing and 72°C extension. The protocol provided by display systems recommends a touch down PCR, but the use of normal PCR resulted in good, clear banding patterns. Treated and untreated roots were run in duplicate for each primer pair to control for anomalies due to cDNA synthesis and PCR.

Polyacrylamide Gel Electrophoresis (PAGE)

To visualize bands, PCR reactions were separated on a 5% polyacrylamide sequencing gel. To make the gel, 18 g of urea was dissolved in 5 ml 10x TBE, 25 ml water and 6.25 ml 40% polyacrylamide solution. Ammonium persulfate was added (0.25 ml of a 10% solution) and the entire mixture was filtered through a 45 micron nylon membrane. The gel mixture was then placed in the refrigerator for 1 to 2 h. The gel plates were then cleaned with soap and water, rinsed with deionized water and wiped with 70% ethanol. The notched plate was coated with approximatly 5 ml Sigmacote (Sigma, St. Louis, MO). The back plate was treated with 100 µl methacryloxysilane, a binding agent. The plates were placed on top of each other with 0.4 mm spacers along the edges and tape was used to seal the bottom and sides. TEMED (35 µl) was added to the chilled gel solution, which was then poured between the plates with a 50 ml syringe. After the gel was poured, the flat edge of a sharks tooth comb was inserted into the top and the gel was allowed to set for 2 h. The comb was removed prior to running and inserted tooth side down into the gel forming wells. The PCR samples were mixed with 3/4 volume of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and 5 µl was loaded into a well. The gel was run at 1500 V for 4-5 h. For fluorescently labeled gels, the gel plates were separated and the gel was immediately scanned on a Storm 860 scanner (Molecular Dynamics, now Amersham, Piscataway, New Jersey).

For radioactive labeled gels, the gel plates were separated and the gel was fixed in 10% acetic acid for 15 min. After rinsing with distilled water, the gel was dried at 37°C overnight. Kodak radiographic film (Fisher Scientific, Pittsburgh, Pennsylvania) was placed directly on the gel and this was placed at –70°C for 24 h. The film was developed on a Konica developing machine (Konica SRX-101, Konica products, Mahwah, New Jersey).

Identification and Reamplification of Differentially Displayed Bands

Bands were scanned by eye and differences in expression determined based upon presence or absence of a band from the duplicate lanes of a treatment. Differentially displayed bands were circled on the radiograph. The radiograph was then laid over the gel and a scalpel was used to dot holes through the film into the gel thus outlining the bands on the gel. These were then cut with the scalpel and placed into 20 µl 5x PCR buffer. The cut bands were heated to 95°C for 15 min and allowed to cool to room temp for an additional 15 min. This solution was then used in PCR to reamplify differentially displayed bands. Reamplification was performed with 1-2 µl of the solution containing

the cut band. The same cycle parameters were used for reamplification except that the annealing temperature was increased to 58°C.

Confirmation of Differential Expression

RNA from orchid roots grown in the presence or absence of the fungus was reverse transcribed and subjected to PCR using gene specific primers. To confirm differential expression, a 1:10 dilution series of each template was made and used in PCR. PCR was performed at 55°C annealing temperature for 35 cycles. Amplified DNA was separated on a 1% agarose gel and visualized using ethidium bromide and UV light.

Analysis of Mycorrhizal Infection

Seedlings of *Cypripedium parviflorum* grown aseptically were transferred under sterile conditions to plastic boxes (Phytamax, Sigma, St. Louis, Missouri) containing sterilized peat perlite (1:1). Cubes (1 cm^2) of oatmeal agar supporting hyphae of *Thanatephorus pennatus* were placed adjacent to the orchid seedlings in the plastic boxes. Fungal hyphae was allowed to overgrow the surface of the peat perlite mix and the roots of the orchid. After 4 weeks, orchid seedlings were removed from the plastic boxes and the roots were excised. Roots were cleared in KOH and stained in chlorazal black (Brundette et al., 1984). Roots and associated fungi were observed under dissecting and compound microscopes.

Results

Differential Display

Seeds of *Goodyera pubescens* were imbibed in water or on oatmeal agar overnight and RNA was extracted to test the differential display protocol. RNA was reverse transcribed and used in differential display analysis. An aliquot of the PCR reaction was run on a 1% agarose gel and product was visualized as a smear with ethidium bromide staining (Fig. 2-1) to confirm the efficiency of PCR. The remaining PCR was separated on a 5% polyacrylamide gel. After electrophoresis, the plates were separated and the gel was wrapped in cling film. The gel was scanned on a Storm scanner reading in the red (Cy5) range. The scanned gel could be immediately visualized on the associated computer. Unfortunately, the detection limit for Cy5 proved too low and bands could not be visualized (Fig. 2-2).

AFLP cDNA Differential Display

RNA was extracted from roots and stems of *Epidendrum radicans* to test the AFLP cDNA method of differential display. The RNA was reverse transcribed and used in AFLP cDNA differential display. After PCR, reactions were separated on 5% polyacrylamide gels, fixed and dried. Dried gels were exposed to x-ray film. In the test run, bands could easily be identified and the bands were consistent for each sample (Fig. 2-3).

Seedlings of *Cyrpripedium* were co-cultivated with *Thanatephorus pennatus* for 12 h to identify orchid genes showing differential regulation in response to a mycorrhizal fungus. This time point was chosen to prevent entry of the fungus into roots of the orchid and thus preclude fungal RNA from analysis. The 12 h time point was also selected to

identify genes that show regulation in the earliest stages of the interaction. RNA from these seedlings was used in the AFLP cDNA differential display. After PCR, samples were separated on a 5% polyacrylamide gel and visualized on radiographic film. Approximately 100 bands (100 - 1000 bp in size) per lane could be seen most of which were the same across treatments (Fig. 2-4). Bands showing differential regulation were cut from the gel and reamplified. Bands that were successfully reamplified at the expected size (Fig. 2-5) were cloned into pGEM T easy (Promega) and sequenced at the Virginia Bioinformatics Institute (Blacksburg, Virginia) sequencing facility. Of about 5000 bands generated by the AFLP, 44 were selected as differentially expressed and cut for reamplification. Of these, only 17 were reamplified, cloned and sequenced. Of those, only 15 produced good sequence data. These sequences were entered into the BLAST program at the National Center for Biotechnology Information web site (NCBI, http://www.ncbi.nlm.nih.gov) and scanned against gene bank. Table 2-2 shows the identity as well as the source and primer pair that generated the fragment of differentially displayed bands. All but two of the sequenced clones showed homology to ribosomal genes. One ribosomal gene was from an *Aspergillus* species and one from the bacterium *Klebsiella pneumoniae* (Schroeter) Trevisan. One clone from untreated roots showed highest homology to trehalose-6-phosphate synthase/phosphatase and was down-regulated. The clone from treated roots showed highest homology to a nucleotide binding protein and was up-regulated.

Confirmation of differential expression was done using PCR with primers specific to the genes of interest. Primers were designed based on the sequence of the differentially displayed gene. A dilution series of RNA from treated and untreated roots was amplified for 32 cycles at 54°C for *NuBP* and 56°C for *Tps*. *Tps* showed differential regulation with expression only in untreated roots and no expression in treated roots (Fig. 2-6A). *NuBP* showed expression to 10^{-3} in treated roots and expression to 10^{-2} in untreated roots suggesting that it had higher expression in the treated sample (Fig. 2-6B)`.

Since *Thanatephorus pennatus* was isolated from *Calypso bulbosa*(L.) Oakes, another species of orchid, it is possible that it is not symbiotic with CyPP and it was necessary to examine its mycorrhizal potential with CyPP. Seedlings grown in axenic culture were transferred to plastic boxes containing sterilized peat perlite (1:1). Cubes (1cm) of *T. pennatus* were added aseptically and allowed to colonize the plants. After 4 weeks, seedlings were removed and examined microscopically for fungal presence in the root. Fungal hyphae were visible as a sheath along the roots of the orchid (Fig. 2-7A). When the fungus was removed the root epidermis came off with it. Hyphae could be seen coiling within cells of the root though most of the cells appeared to be epidermal (Fig. 2-7B). No fungus could be seen in the cortex and no pelotons were visible. However, no signs of necrosis could be observed either.

Discussion

Differential display has become a well-established technique to identify genes that show regulation during growth or in response to some external factor. Though there seem to be many successes with this technique it is not without problems and the rate of return of useful information is low. In the current study, traditional differential display failed to generate any data. This could be due to the use of fluorescent-labeled primers

and associated laser scanning to detect expressed genes. Though the detection limit for fluorescent-tagged cDNA is supposed to be in the sub-nanomolar range, bands could not be visualized. Amplification did seem to be occurring as checks of amplification on agarose gels showed a smear of DNA in the expected size range. Since the oligo dT primer is the source of the label it could be that this primer is not being incorporated into product and that the random primer is generating most of the fragments. Fluorescent differential display has enabled other researchers to identify circadian clock regulation in *Arabidopsis* (Kreps et al., 2000) and cytokinin induced gene expression in tobacco (Kimura et al., 2001). However, Jones and Harrower (1998) found that differential display was not a good technique to identify gene changes brought about by nematode infection in potato. Because of the limit in detection in this study, it is not possible to further determine the efficacy of a standard differential display protocol.

Since many problems have been associated with differential display (Debouck, 1995), attempts have been made to better the technique. One such method was introduced by Bachem et al. (1996) and involves the use of amplified fragment length polymorphisms (AFLPs). The use of AFLPs depends on digestion of DNA with restriction enzymes and ligation of double stranded adaptors to the cut ends of the DNA. Because the sequences of the adaptors are known, it is possible to increase the stringency of PCR as primers are designed to bind to the adaptors. This improves on standard differential display, which has low stringency PCR and uses primer miss-matches to increase product (Appel et al., 1999). This low stringency PCR could favor the amplification of common cDNAs with low match to the primer over uncommon sequences with good match to the primer. The AFLP based cDNA differential display introduced by Bachem et al. (1996) uses two restriction digests to select a subsample of the cDNAs. They used a rare cutter to cut each cDNA once and a second frequent cutter that would cut on one or both sides of the rare cutter. Using this method they estimated that they could amplify approximatly 40% of the expressed genes in an experimental system. Habu et al. (1997) used only a frequent cutter to generate fragments for display analysis, a method which would select nearly all cDNAs.

The AFLP differential display protocol followed in this study uses only the single cutter to generate fragments for analysis. However, two separate adaptors are ligated to the cut ends of the fragment. Since only a single cutter is used, the ligation site is the same for both adaptors and there is no control over which adaptor binds where. To control partially for this, one adaptor (the 0-extension adaptor) has an alteration to prevent primer binding or primer extension during the first round of PCR. This means that fragments which happen to have this adaptor annealed at both ends are not amplified. The primer specific to the other adaptor (probe primer) has a 3 bp overhang that extends into the fragment. Since it is highly unlikely that the sequence adjacent to two opposing TaqI sites would be identical, amplification of fragments with this adaptor in opposing orientations is also unlikely. It is still possible that adaptors could ligate differently in each sample. To control for this, duplicate samples of each RNA sample were processed through the cDNA synthesis and adaptor ligation steps.

Amplified bands showing apparent differential expression were easily identifiable using the AFLP cDNA differential display method and duplicate samples were nearly identical. Problems were noticeable in the strength of signal between duplicate samples, which can be explained only by inconsistencies in pipetting due to aerosols, fluid

retention, and pipettes that are out of calibration. Bands selected as differentially expressed were chosen only if there was a clear change in signal strength between bands of different samples. Though 44 bands were cut from the gel, only 17 of these were successfully reamplified (as clear, distinct bands). As others have mentioned, the failure to reamplify could be due to misalignment of the radiograph with the gel during cutting of the bands (Wan et al., 1996). Since amplified DNA was not purified from the gel, contaminants such as urea may have hindered subsequent PCR. Some samples that could not be reamplified showed a smear of PCR product after reamplification. This could be due to the fact that a single band may contain more than one cDNA species. The fact that less than 50% of bands were reamplified indicates a possible limitation of this protocol. However, two of the bands that were reamplified were done so only after performing a second AFLP differential display and extracting the band from a second gel.

That 13 of the 15 sequenced differentially expressed bands were identified as ribosomal RNAs is unexpected but not surprising (Nagel et al., 2001). Isolation of mRNA from total RNA was not performed and RT conditions were not stringent, which means that the oligo dT primer could have easily annealed to poly A tracts within ribosomal RNAs. Furthermore, since northern blot analysis was not performed, these genes may represent the false positives reported in other research (e.g. Benito et al., 1996) using differential display. However, the AFLP method is supposed to cut down on the number of false positives. It is of interest that most of the RNA genes were found in orchid roots grown with the fungus and may suggest that the cell is preparing for large-scale protein synthesis in response to the fungus.

Two of the sequenced bands showed highest homology to genes from organisms other than plants, ribosomal RNA from *Klebsiella pneumoniae* and ribosomal RNA from an *Aspergillus* species. The gene from *K. pneumomiae* was found in orchid roots grown with the fungus whereas the *Aspergillus* species was found in orchid roots grown without the fungus. Since plants were not grown aseptically, they were exposed to microorganisms present in the lab and it is therefore conceivable that genes of these two microorganisms can be present in AFLP cDNA differential display analysis. That the two were differentially expressed could point to the strength of this protocol as it is possible that only one plant within a sample pool could be contaminated with the organism and this would lead to differential expression.

Of the 42 bands selected to be differentially expressed, only two were non-ribosomal. These two genes were also confirmed to be differentially expressed using PCR. One gene (nucleotide binding protein) was up-regulated while the other (trehalose-6-phosphate synthase phosphatase) was down-regulated. Both genes may be involved with fungal interactions. The protein product of *Tps* is involved in synthesis of trehalose, a fungal sugar. Trehalose has been shown to interact with sugar metabolism, carbon partitioning and sugar signaling. Alterations in trehalose synthesis could thus promote carbon partitioning to the roots which would be beneficial to the fungus. Likewise, altered sugar signaling due to changes in trehalose levels could lead to altered growth patterns which could be necessary to formation of the symbiosis. The protein NuBP is closely related to the *E. coli* (Migula) Castellani and Chalmers minD class of partitioning ATPases. These enzymes are believed to play a role in cell division and have been shown to be essential in yeast. In orchids, an increase in *NuBP* could indicate an increase in growth concomitant with an increase in cell division. This increased growth

could be part of the response to the formation of the mycorrhiza. It is also possible that an increase in cell number is necessary to accommodate the fungus within the root. It is interesting to note that *Aspergillus* present in untreated plants did not trigger the regulation of either gene suggesting that both genes are responsive to the mycorrhizal fungus.

The use of differential display led to the identification of two genes associated with mycorrhiza in roots of a native orchid (*Cypripedium parviflorum* var. *pubescens*). An initial attempt using fluorescence differential display failed to yield any results due to the detection limit of the fluorescent dye. An AFLP based method was much better generating about 5000 fragments. Many of the genes selected as being differentially expressed turned out to be ribosomal genes. This may have been due to the procedure used to generate fragments or could reflect changes in protein production in response to the fungus. The genes identified as differentially expressed, trehalose-6-phosphate synthase/phosphatase and nucleotide binding protein, need to be characterized further before their role in orchid mycorrhizal interactions can be determined.