Table 2-1. Random upstream primers used in fluorescence differential display.

| Upstream primer[a] | Sequence |
|---|---|
| 1 | 5’ GATCATAGCC |
| 2 | 5’ CTGCTTGATG |
| 3 | 5’ GATCCAGTAC |
| 4 | 5’ GATCGCATTG |
| 5 | 5’ AAACTCCGTC |
| 6 | 5’ TGGTAAAGGG |
| 7 | 5’ GATCATGGTC |
| 8 | 5’ TTTTGGCTCC |

a. Primers were purchased from Display Systems Biotech and are listed numerically to differentiate them

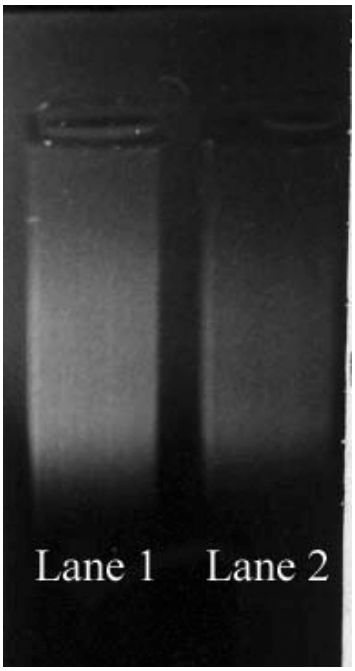


Figure 2-1. Agarose gel analysis to confirm amplification of cDNA from imbibed and non-imbibed seeds of *Goodyera pubescens* using differential display PCR.
Seeds of *Goodyera pubescens* were either imbibed in water for 24 h or left unimbibed prior to RNA extraction. RNA was reverse transcribed with an oligo 18dT primer and the resultant cDNA was used in differential display PCR. The presence of amplified DNA following PCR was verified by agarose gel analysis. An aliquot of the PCR was subjected to electrophoresis on a 1% agarose gel and visualized using ethidium bromide and UV light. The multiple cDNA fragments generated by differential display PCR are visualized as a smear on the agarose gel, indicating that differential display PCR is generating fragments. Lane 1: unimbibed seeds amplified using the downstream primer and upstream primer 1. Lane 2: imbibed seeds amplified using the downstream primer and upstream primer 1.

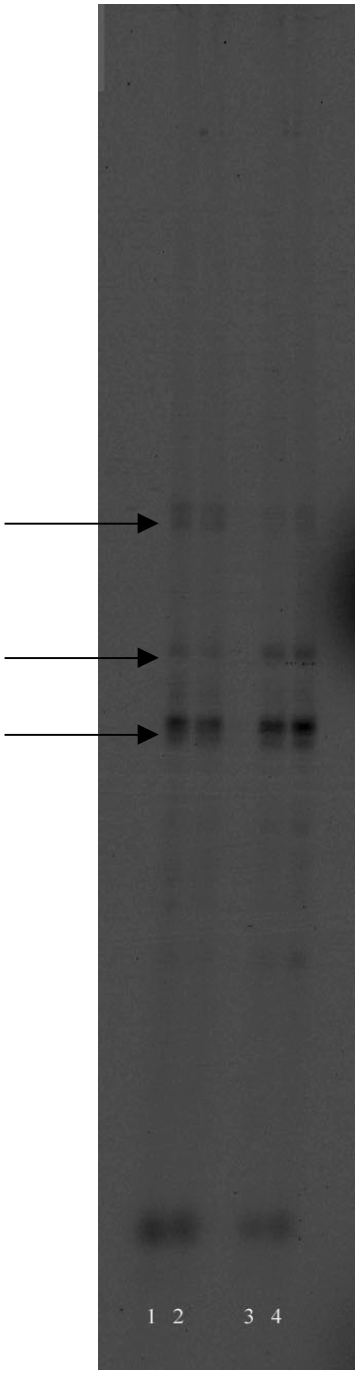


Figure 2-2.

Figure 2-2. Fluorescence differential display of imbibed and non-imbibed seeds of *Goodyera pubescens*.
Seeds of *Goodyera pubescens* were either imbibed in water for 24 h or left unimbibed prior to RNA extraction. RNA was reverse transcribed with an oligo 18dT primer and the resultant cDNA was used in differential display PCR. Differential display products were separated on a 5% polyacrylamide gel. Visualization of the Cy5 fluorescent dye linked to the downstream primer was accomplished using a Storm scanner. Lane 1 and 2: non-imbibed seeds amplified with the downstream primer and upstream primer 1. Lane 3 and 4: imbibed seeds amplified with the downstream primer and upstream primer 1. Arows indicate amplified cDNAs. Only a few of the many cDNA fragments were visualized by fluorescence indicating that, in this instance, fluorescence is not suitable for detection of cDNA fragments.

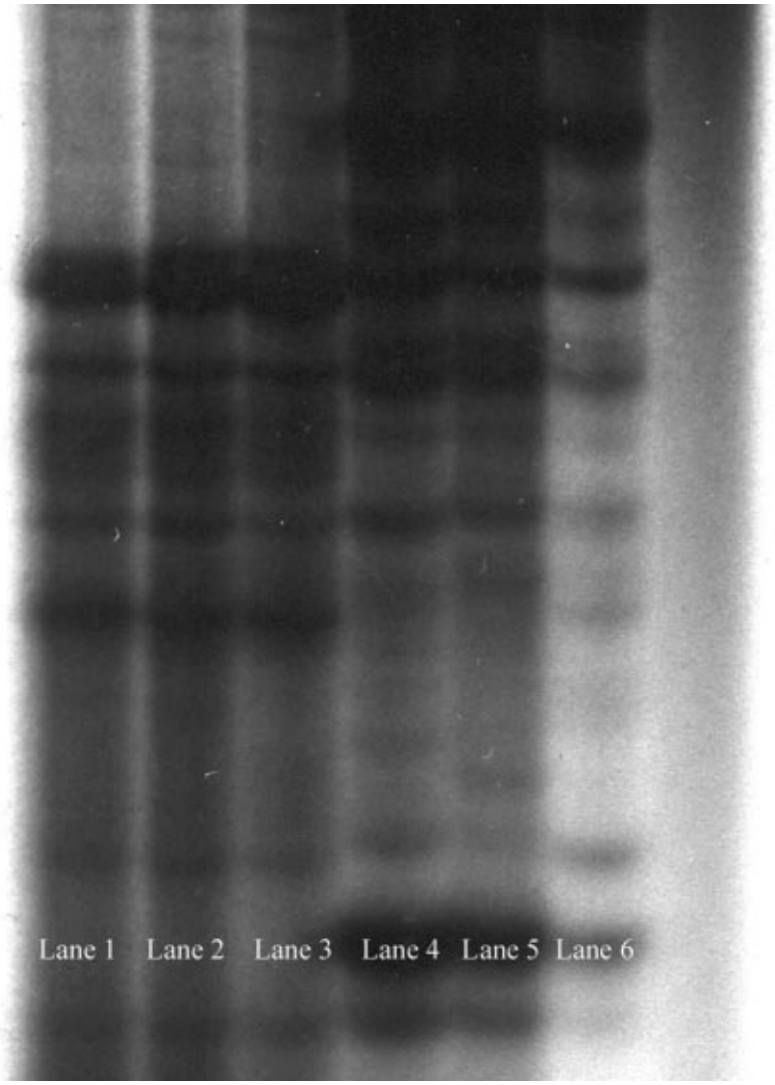


Figure 2-3. Autoradiogram of test of AFLP-DD using roots of *Epidendrum radicans*. Roots of *Epidendrum radicans* were excised from plants growing in the greenhouse and RNA was extracted. RNA was reverse transcribed and digested with *TaqI* restriction enzyme. Adapters were ligated to the cDNA and the resultant template was used in PCR. Two primers, an adapter primer and one of 64 probe primers, specific to the adapters were used to amplify cDNA by PCR. Amplified, radiolabeled cDNA was separated on a 5% polyacrylamide gel and visualized by exposure to X-ray film. Lanes 1-3: PCR amplification of cDNA of *Epidendrum radicans* with the adapter primer and probe primer 1. Lanes 4-6: PCR amplification of cDNA of *Epidendrum radicans* with the adapter primer and probe primer 2. The concentration of dNTPs was 25 μM (Lanes 1 and 4), 50 μM (Lanes 2 and 5), and 100 μM (Lanes 3 and 6). Multiple cDNA fragments can be observed indicating that radioactive labeling is suitable for detection of cDNA fragments.

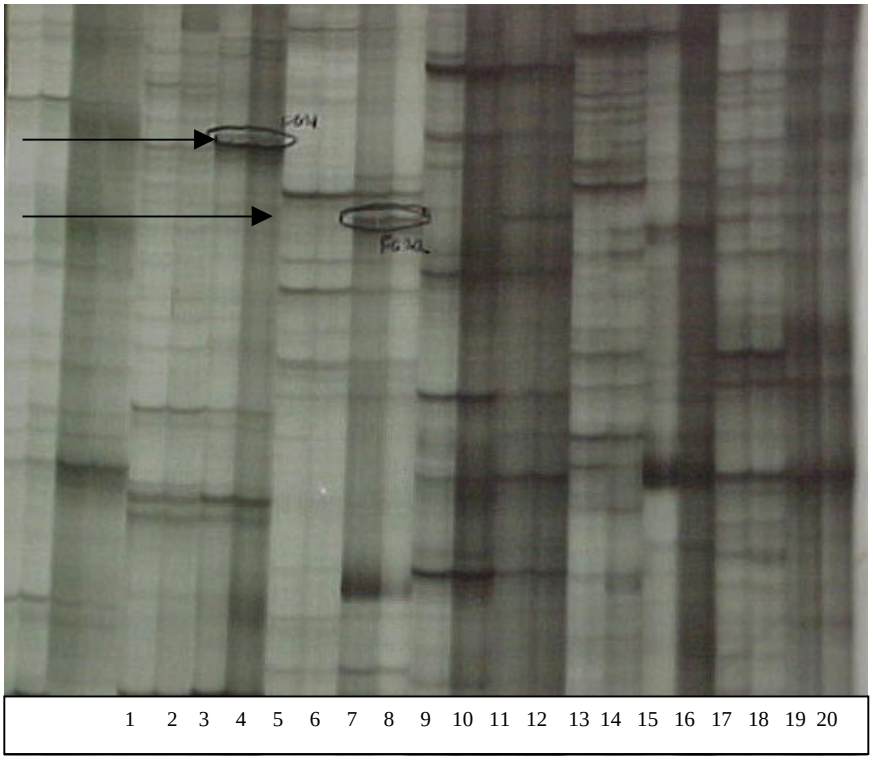


Figure 2-4. Autoradiogram of AFLP DD using roots of *Cypripedium parviflorum* grown in the presence or absence of *Thanatephorus pennatus*.
Seedlings of *Cypripedium parviflorum* were grown with *Thanatephorus pennatus* for 12 hours or left untreated prior to RNA extraction. Extracted RNA was reverse transcribed and the resultant cDNA was digested with *TaqI* restriction enzyme. Adapters were ligated to the cDNA and the resultant template was used in PCR. Two primers, an adapter primer and one of 64 probe primers, specific to the adapters were used to amplify DNA by PCR. Amplified, radiolabeled cDNA was separated on a 5% polyacrylamide gel and visualized by exposure to X-ray film. Each group of 4 lanes represents one set of primers: Lanes 1-4, adapter primer and probe primer 4; Lanes 5-8, adapter primer and probe primer 5; Lanes 9-12, adapter primer and probe primer 6; Lanes 13-16, adapter primer and probe primer 7; Lanes 17-20, adapter primer and probe primer 8. The first two lanes of each group of 4 represent duplicate samples of amplified cDNA from untreated roots and the second 2 lanes represent duplicate samples of amplified cDNA from treated samples. The multiple fragments generated by AFLP-DDPCR can be seen. Arrows indicates potentially differentially expressed fragment.

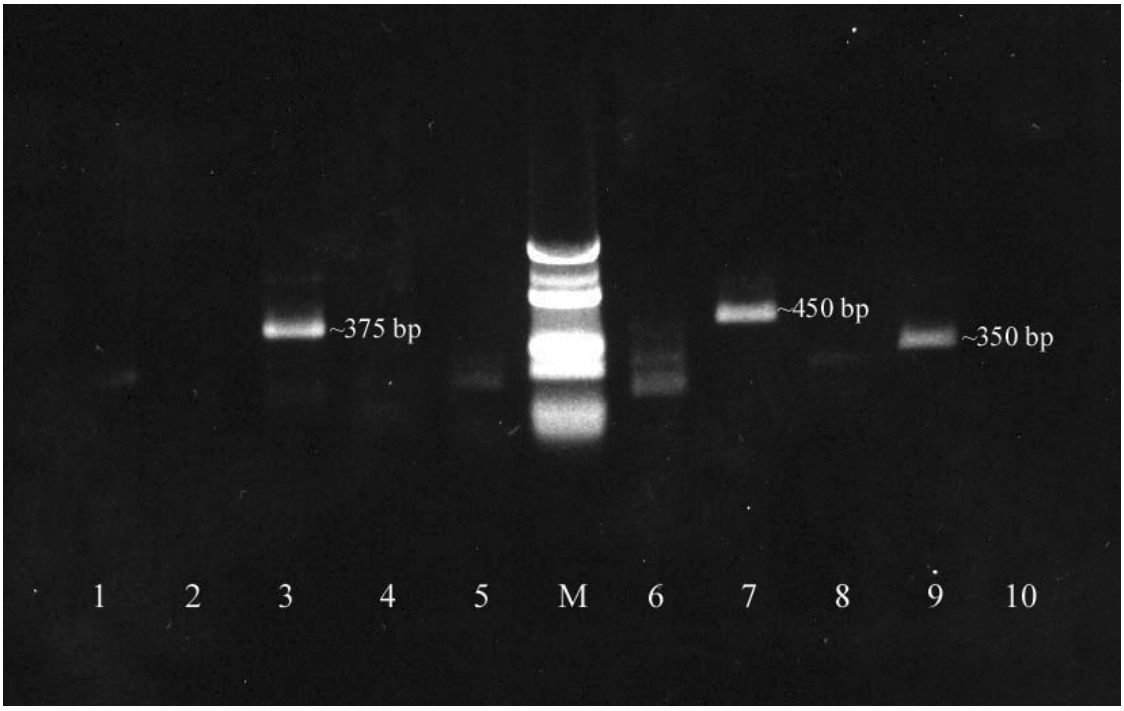


Figure 2-5. Reamplification of differentially expressed genes.
Fragments showing differential regulation were isolated from differential display gels and DNA was eluted in water. An aliquot of the eluted DNA was then reamplified using PCR. The primers used for reamplification were the same as those used to generate the band of interest in the AFLP-DD PCR (the adapter primer in combination with one of the probe primers). The re-amplified fragments were separated on a 1% agarose gel and visualized with ethidium bromide staining and UV light. Reamplified fragments are from control, (roots of *Cypripedium parviflorum* grown in the absence of *Thanatephorus pennatus*) and treated (roots of *C. parviflorum* grown with *T. pennatus*) samples. Numbers refer to the probe primer used in combination with the adapter primer to generate fragments. Lane 1: control 17A, Lane 2: control 19A, Lane 3 treated 5A, Lane 4: treated 9A, Lane 5: treated 9B, Lane 6, control 10B, Lane 7: control 11A, Lane 8, control 11B, Lane 9: treated 13A, Lane 10, treated 13B. Some fragments were succesfully reamplified (Lane 3), others were not reamplified (Lane 2), still others generated more than one band (Lane 6) suggesting multiple fragments exist in some differentially expressed bands.

Table 2-2. Putative identities of genes isolated from AFLP-cDNA differential display.

| *Source* | *Fragment Number* | *BLAST Match* |
|---|---|---|
| Orchid only | 1 | No significant alignment with known genes |
| Orchid only | 2 | Trehalose phosphate synthase/phosphatase, Rice |
| Orchid only | 3 | 26S large subunit ribosomal RNA gene |
| Orchid only | 4 | No significant alignment with known genes |
| Orchid only | 5 | Fungal mitochondrial DNA for small ribosomal subunit, *Aspergillus sp*. |
| Orchid + fungus | 6 | Mitochondrial DNA similar to 26S RNA |
| Orchid + fungus | 7 | Mitochondrial 26S ribosomal RNA gene |
| Orchid + fungus | 8 | Mitochondrial 26S ribosomal RNA gene |
| Orchid + fungus | 9 | Nucleotide binding protein, *Arabidopsis* |
| Orchid + fungus | 10 | Mitochondrial gene for small ribosomal RNA gene |
| Orchid + fungus | 11 | Gene for 18S ribosomal RNA |
| Orchid + fungus | 12 | Bacterial DNA for 23S ribosome, *K. pneumoniae* |
| Orchid + fungus | 13 | Gene for 18S small ribosomal subunit |
| Orchid + fungus | 14 | Gene for 18S small ribosomal subunit |

The RNA source from which the differentially expressed gene was isolated is indicated as is the number of the fragment isolated as being differentially expressed. Possible function was determined from the highest scoring match using BLAST search at the National Center for Biotechnology Information website. The organism with which the fragment had the highest match is listed where known.

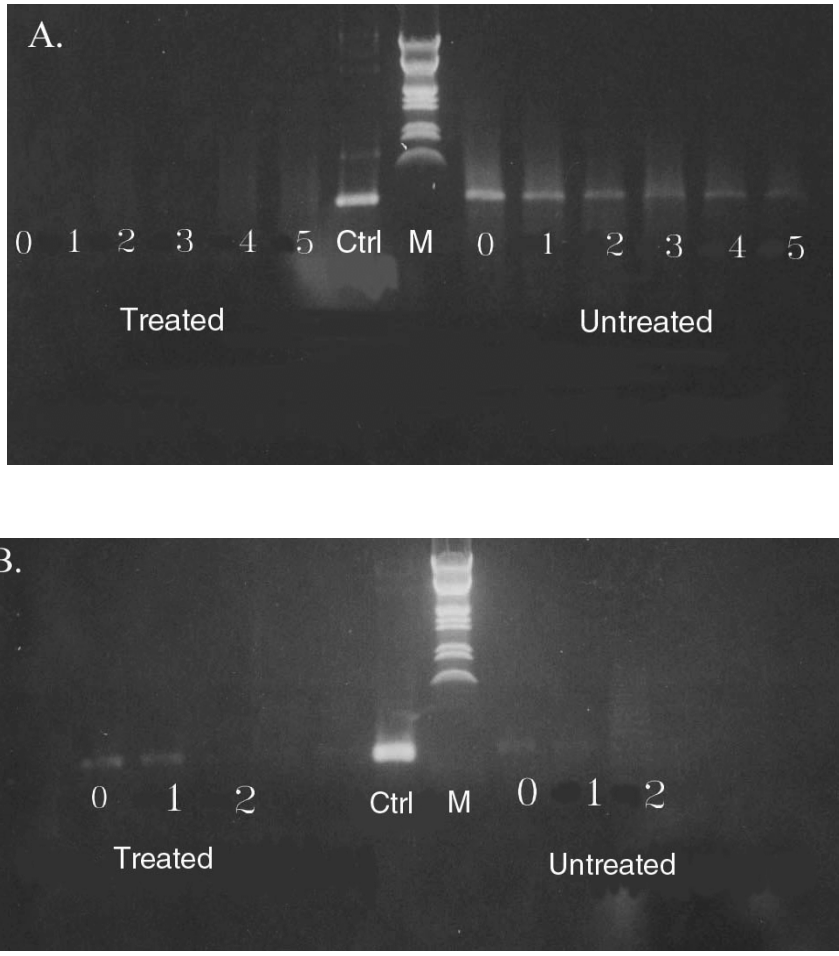


Figure 2-6. Confirmation of differential expression of *Tps* and *NuBP*.
RNA from roots of *Cypripedium parviflorum* grown in the presence (treated) or absence (untreated) of *Thanatephorus penatus* was reverse transcribed. To confirm differential expression, a 1:10 dilution series of each cDNA was made and used in PCR. PCR was performed at 55°C annealing temperature for 35 cycles with gene specific primers for both *NuBP* and *Tps*. Amplified DNA was separated on a 1% agarose gel and visualized using ethidium bromide and UV light. Ctrl is the positive control. M is the molecular size marker.

A. Confirmation of differential expression of *Tps*. Numbers refer to the log base 10 of the dilution factor. Expression of *Tps* is greater in untreated samples.
B. Confirmation of differential expression of *NuBP*. Numbers refer to the log base 10 of the dilution factor. Expression of *NuBP* can be seen to be slightly higher in treated samples.

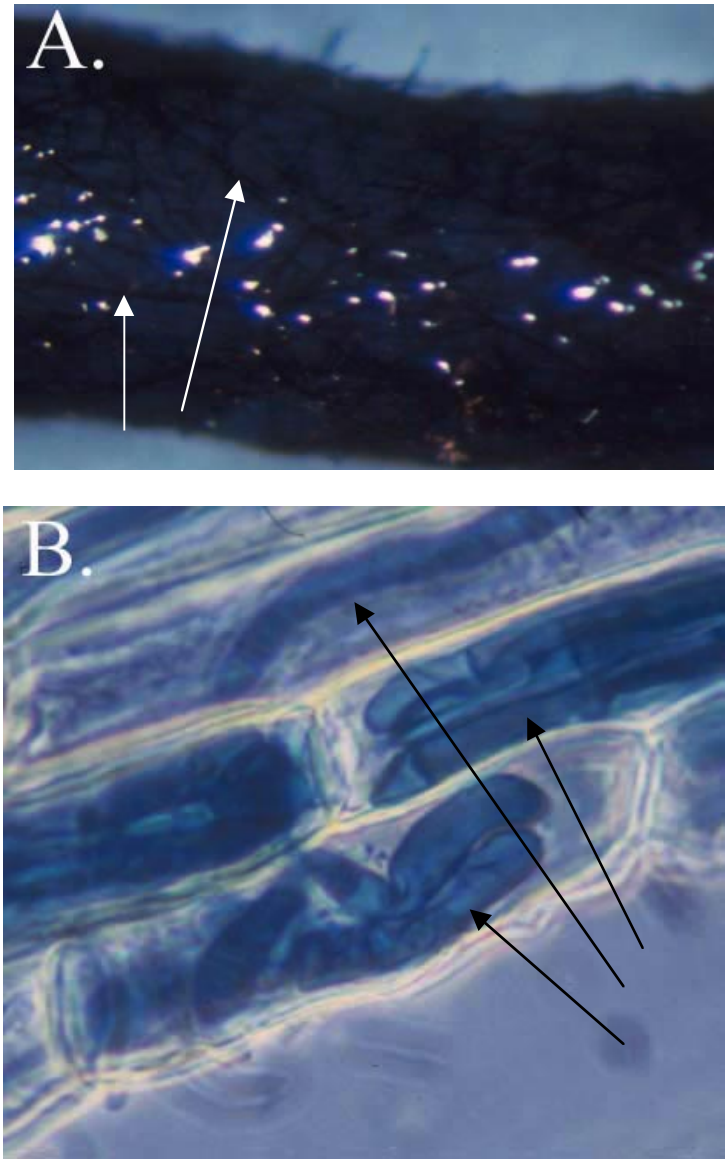


Figure 2-7. Analysis of mycorrhizal formation between *Cypripedium parviflorum* and *Thanatephorus pennatus*
Seedlings of *C. parviflorum* were obtained in sterile culture from a commercial grower. The seedlings were transferred to plastic culture boxes containing sterilized peat:perlite (1:1). Cubes of *T. pennatus* were added under sterile conditions. After 4 weeks, plants were removed from the boxes and roots were excised. Roots were then stained with chlorazal black and photographed.

A. Root of *C. parviflorum* showing hyphae of *T. pennatus* covering the epidermis. Photographed on a dissecting microscope. Hyphal stands can be seen as dark threads covering the root (arrows).
B. Squashed section of root of *C. parviflorum* showing epidermal cells and cortical cells infected with fungi. Fungi can be seen as darker blue sausage-shaped cells (arows) within the tabular cells of the orchid. Photographed on a compound microscope.