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*Full Length Research Paper*

# Screening and cloning of differentially expressed genes in *Dendrobium nobile* induced by orchid mycorrhizal fungus promoting the growth

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**Appropriate mycorrhizal fungi could effectively promote plant growth and development. Our previous research results showed that the growth of *Dendrobium nobile* was obviously promoted under inoculating one orchid mycorrhizal fungi, *Epulorhiza* sp. AR-18. To understand the growth-promoting molecular mechanisms, differential displayed real time polymerase chain reaction (DDRT-PCR), reverse Northern blot and Southern blot were used to isolate and identify genes whose transcription were altered in cultured *D. nobile* plants that were treated with *Epulorhiza* sp. AR-18. Amplified by 8 primer combinations from one anchor primers and 8 random primers, a total of 14 complementary DNA (cDNA) fragments including 12 differentially expressed cDNA bands were isolated. Reverse northern blot analysis showed that only 2 genes were differentially displayed cDNA bands. One band was an especially expressed fragment, expressed in the treated group but not in the control; while another was a differentially expressed fragment, weak in the treated and strength in the control. Southern blot analysis demonstrated that the two gene fragments were from the plant and not from the fungus. Sequence analysis and database searches revealed no significant homology to any known sequences. The results suggested that the usefulness of messenger RAN (mRNA) differential display technique for the detection of differentially expressed genes in *D. nobile* whose growth could be promoted by mycorrhizal fungi.**

**Key words:** *Dendrobium nobile*, differential displayed real time polymerase chain reaction (DDRT-PCR), orchid mycorrhizal fungus, *Epulorhiza* sp., reverse northern blot.

## INTRODUCTION

The traditional Chinese crude drug 'Shihu', derived from the dried or fresh stems of several *Dendrobium* species (Orchidaceae), is widely used as both traditional Chinese and folk medicines for the treatment of various diseases, such as chronic atrophic gastritis, diabetes, skin aging and cardiovascular diseases, which are believed to be closely associated with the metabolic disorders of reactive oxygen species (ROS) and the promotion of body fluid in the human body (Zhang et al., 2008a). *Dendrobium nobile* Lindl. is one of the most popular *Dendrobium* plants and has been recorded in the Chinese Pharmacopoeia (2005 Edition) as one of the original materials of 'Shi Hu' (Zhang et al., 2008b).

In recent years, the living environments of *D. nobile* were heavily destroyed and its wild sources were faced to extinction because of constant increasing for use and excessive exploitation in the producing areas. So far, the artificial cultivations of *D. nobile* in large areas could not be successfully obtained as for harsh ecological environments. All plants of Orchidaceae are symbiotic with fungi under field conditions (Lawrence and Cara, 1998). Many mycorrhizal fungi were isolated from the roots of wild *D*.

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*candidum* and *D. nobile* collected from Yunnan and Sichuan provinces and screened out three strains helpful to the growth and development of *Dendrobium* in our laboratory for many years.

Among the three strains, AR-18, a species of genus *Epulorhiza*, was tested obvious influence to the fresh weight and dry weight of *D. nobile*. In vermiculite medium, *Epulorhiza* sp. AR-18 significantly increased the fresh weight and dry weight in *D. nobile.* The fresh weight increased by 16% and the dry weight by 21% compared with the non-inoculated one (Song and Guo, 2001). MF18, which was *Epulorhiza* sp. AR-18, could increase the content of polysaccharides by 52.1%, and another fungus, MF23, also belonging to genus *Epulorhiza*, was shown to advance contents of polysaccharides by 18.5% and total alkaloids by 18.3% compared with the respective control groups in *D. nobile*. Differential displayed real time polymerase chain reaction (DDRT-PCR) is a powerful technique used to identify and isolate genes rapidly that are differentially expressed for plants under biotic and abiotic stress (Venkatesh et al., 2005; Venkatachalam et al., 2009). In this paper, the DDRT-PCR technique was applied to explore how the orchid mycorrhizal fungus affected the gene expression in *D. nobile*.

## MATERIALS AND METHODS

The axenic *D. nobile* seedlings were cultured on 1/2 MS medium (Murashige and Skoog, 1962), including extra addition of appropriate amounts of potato extracts (200 $gdm^{-3}$) and sucrose (30 $gdm^{-3}$). The mycorrhizal fungus strain (No: AR-18), an *Epulorhiza* sp. entophytic fungus, was previously identified and exhibited growth-promoting effects on *D. nobile* (Song and Guo, 2001). The strain was maintained on the plate of wheat bran medium, containing 30 g $dm^{-3}$ wheat bran, 1.5 g $dm^{-3}$ $MgSO_4$, 3 $gdm^{-3}$ $KH_2PO_4$, 20 g $dm^{-3}$ D-glucose, and 14 g $dm^{-3}$ agar (pH 5.8). The fungus stored at 4°C was cultured to be activated for 10-15 days at 24 ± 1°C in darkness, then ready for platelets preparation by a puncher (Φ11mm), which was then used as the inoculums.

Inoculation was performed in aseptic condition following the procedures previously described by Song and Guo (2001). Briefly, a small quantity of the sterilized splinters of robur leaves, referred to as a holder, was added into the bottom of a sterile triangular flask (100 ml), and two fungal platelets were simultaneously put onto the splinters. The axenic *D.* seedlings were then transferred on the holder around the fungal platelets, followed by filling the *D. nobile* roots with the mixtures of vermiculite and grit (3:2), and pouring with 11 ml sterile distilled water for each flask. The mock inoculation without fungal inoculum but the holders including the vermiculite and grit mixtures was used as the control. The treated groups and the control groups were inoculated ten triangular flasks, respectively. All the plants were co-cultured in a growth chamber at 24 ± 1°C. The control and fungal inoculated seedlings were harvested 90 days post inoculation (dpi). All the aerial parts including the stem and leaf tissues were collected, and immediately frozen in liquid nitrogen, then stored at -80°C for RNA isolation.

Total RNA was isolated from 2 g aerial parts of the fungal inoculated and the control seedlings using improved CTAB method described by Chang et al. (1993). Total RNA was then treated with $RQ_1$ Rnase-free DNase I (Promega, USA) to eliminate DNA contamination. The RNA pellets in ethanol were stored at -80 °C until used. Ten microgram total RNA was treated with 10 units of $RQ_1$ Rnase-free DNaseI (Promega, USA) in 10 µl reaction volumes at 37°C for 30 min followed by 65°C incubation for 10 min. DNase I treated RNA samples were precipitated in 2.5 M LiCl by incubating at -20°C and collected by centrifugation at 4°C, 12,000 g for 15 min. The RNA pellets were washed in 70% ethanol and suspended in 10 µl DEPC-treated $ddH_2O$ just before use. The quality and integrity of total RNA were determined by running 1 µl of total RNA in a formamide denaturing gel, and the quantity was tested using a Hitachi 2000 spectrophotometer (Hitachi, Japan).

The first strand complementary DNA (cDNA) was synthesized using M-MLV Reverse Transcriptase (GiBco-BRL). cDNA constructions were performed according to the manufacturer's instructions as follows: 4 µl total RNA (10 µg) was incubated in the presence of 4.5 µl 0.1 µg $µL^{-1}$ anchor primer (5′-AAGCTTTTTTTTTTG-3′) and sterile $ddH_2O$ in 20 µl total volume at 70°C for 5 min and set on ice. The synthesis was performed in 8 µl 5× first strand buffer (GiBco-BRL), 4 µl 0.2 mM dNTPs, 4 µl 0.1 M DTT (GiBco-BRL), 2 µl 200 U $µL^{-1}$ M-MLV Reverse Transcriptase (GiBco-BRL) and 2 µl 30 U $µL^{-1}$ RNase inhibitor (Sangon, Shanghai, China) in 40 µl total volume, incubated for 90 min at 50°C and stopped at 70°C by 15 min incubation.

DDRT-PCR was performed on single-strand DNAs using eight primer sets with the anchor primer as used in RT in combination with eight random primers (B0303, 5′CTTTCTACCC3′; B0304, 5′TTTTGGCTCC3′; B0306, AAACTCCGTC3′; B0311, 5′TACCTAAGCG3′; B0314, 5′GATCAAGTCC3′; B0318, 5′GATCTCAGAC3′; B0320, 5′GATCAATCGC3′; B0321, 5′GATCTAACCG3′; Sangon, Shanghai, China) (Bauer et al., 1993). cDNAs (diluted 10 folds) from fungal and mock inoculated samples were combined to be used in DDRT-PCR experiments and amplified with 0.5 µl 5 U $µL^{-1}$ Taq polymerase (Sangon, Shanghai, China) in the presence of 2 µl 10 × PCR Buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.8, 0.1% Triton X-100), 2.5 µl 25 mM $MgCl_2$, 2 µl 10 mM dNTPs (Sangon, Shanghai, China), 2 µl 5 µM anchor primer as used in RT, 2 µl 5 µM 10-mer random primer and sufficient sterile distilled water up to 20 µl volume. Thermal conditions were 95°C for 3 min followed by 35 cycles of 94°C for 30 s, 40°C for 2 min, and 72°C for 30 s, with a final 72°C for 7 min.

PCR products were separated on a standard DNA denaturing sequencing gel (6% denaturing gel, 5.7% acrylamide, 0.3% N, N' methylene-bis-acrylamide, 8 M urea, 1× TBE). Prior to loading, 4 µl of each PCR product mixed with 2 µl of loading dye (95% formamide, 10 mM EDTA, pH 8.0, 0.01% bromophenol blue) was denaturated at 95°C for 2 min. Silver Staining of the polyacrylamide gel was performed as described by Zhang et al. (1997), with minor modifications that 7.5% acetic acid (v/v) was used for fixing the gel immediately after electrophoresis and terminating the following developmental reaction. The fragments determined as differentially displayed bands were excised from the gels using a clean razor blade and suspended in 20 µl $ddH_2O$. The mixtures were then incubated in a water bath at 90-100°C for 15 min, followed by centrifugation at 10,000 g for 5 min. The resulting supernatant containing the isolated DNA fragments was therefore used directly as the template for reamplifying the corresponding DNA fragments, using the identical PCR thermal conditions as abovementioned. Products of the reamplified bands were screened on 1.2% agarose gel to ensure the correct size of each fragment that was specifically amplified.

To confirm the origin of differential fragments, we conducted southern blot analysis following the standard procedures (Sambrook et al., 1989). Total genomic DNA of *D. nobile* and AR-18 were respectively extracted by CTAB method (Stewart and Via, 1993). Each five microgram DNA was then digested by three endonuclease *BamH* I, *EcoR* I and *Pst* I and separated on 0.8% agarose gel in TAE buffer, and transferred onto Hybond $-N^+$ Nylon membranes (Amersham Biosciences, Little Chalfont, UK). The reamplified cDNAs fragments were radio-labeled in regular PCR with 1 U Taq DNA Polymerase (Sangon, Shanghai, China) in the presence of 20

ng template of each cDNA, 1 μM anchor primer, 1 μM random primer, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 50 μM dATP, 50 μM dGTP, 50 μM dTTP, 0.5 μM α-$^{32}$P-dCTP (specific activity 10 μCi/μl) and sterile distilled water up to 20 μl volume. Hybridization was then performed with the corresponding α-$^{32}$P-dCTP labeled DNA probes according to a published protocol (Church and Gilbert, 1984), and the membranes were washed with 2× SSC containing 0.1% SDS at room temperature (22-24°C) for 10 min once and with 2× SSC containing 0.1% SDS at 65°C for 10 min. Then membranes were dried and visualized with a phosphor imager (Fujifilm, Japan).

Reverse Northern blotting analyses were employed to confirm the differential plant transcript that was truly induced by mycorrhizal fungus infection (Rosa et al., 2007). All the differential fragments of re-amplification products were loaded on the gel and transferred onto Nylon membranes as abovementioned description. cDNAs reverse transcribed from the fungal infected and control plant samples were used as hybridization probes and they were radio-labeled also with α-$^{32}$P-dCTP by reverse transcription according to the protocol described by (Church and Gilbert, 1984). Hybridization was performed according to Zhang et al. (1997) under high-stringency conditions.

The positive hybridization bands from blotting analyses were therefore subjected to cloning and sequencing analyses. Each of 5 μl reamplified product of the corresponding positive hybridization bands was ligated into 50 ng pBluescript Ⅱ SK(+/-) T-vector (Sangon, Shanghai, China) in 10 μl reaction volume in the presence of 6 units of T4 DNA ligase and 1× ligase buffer at 4°C for overnight. The ligation products were transformed into component *E. coil* strain Dh5-α. Three white colonies were randomly selected and the presence of insert was confirmed by endonuclease digestion before sending to Shanghai Sangon Co. Ltd. for sequencing. Sequence similarity analyses were finally conducted using BLAST analysis searching against nr database in Genbank at NCBI website.

## RESULTS

The differential display technique was applied for the identification and isolation of genes whose transcription was differentially regulated by the entophytic fungi on *D. nobile*. Eight 5′ random primers were used for PCR amplification in combination with one 3′ anchored primer and used to amplified cDNAs obtained by reverse transcription of total RNA from the control and the treated groups. To avoid isolation of false positives, all PCR reactions were repeated three times for each group. Only cDNAs which could display stably were selected for further analysis. The PCR products were separated on 6% denaturing polyacrylamide gel electrophoresis (PAGE) gel and be silver staining. Figure 1 shows a representtative gel from a differential display experiment. Fourteen differentially expressed cDNA bands, dd-1, dd-2, dd-3, ~, dd-14, respectively were detected in total and eluted successfully from the polyacrylammide gel. The differential cDNA bands showed an increased or decreased band intensity following mycorrhizal fungi infection in comparison to the corresponding bands in control.

Each fragment for all 14 recycled cDNA bands was reamplified by PCR using the primers that yielded the original band. By three times PCR amplification, twelve PCR products of the recycled bands met the requirements of hybridization, which were dd-1, dd-2, dd-4, dd-5, dd-6, dd-7, dd-8, dd-9, dd-11, dd-12, dd-13, dd-14 band, respectively. To confirm whether the 12 recycled bands were true differences or artifacts due to the PCR amplification process, reverse Northern dot-blot analyses were performed (Figure 2). In this analysis, only two (dd-1 and dd-8) bands were the differentially expressed ones. The band dd-1 using primer pairs, the anchor primer and random primer B0303, was an especially expressed cDNA band which was not in the control groups but was in the treated groups by the fungus. The band dd-8 amplified by the primer pairs, the anchor primer and random primer B0318, was a differentially displayed cDNA band which was expressed more intensive in the control than in the treated. The 4 bands, dd-6, dd-11, dd-12 and dd-14 were non differential bands, which had a same expressed intensity in both groups. The 6 bands, dd-2, dd-4, dd-5, dd-7, dd-9 and dd-13 were all not displayed in both groups and therefore also false positive bands.

The genome DNA of *D. nobile* and Fungus AR-18 were digested by three endonucleases BamH Ⅰ, EcoR Ⅰ and Pst Ⅰ, respectively. The cutting products of each endonuclease were isolated and detected on the agarose gel (Figure 3A). The digested DNA of *D. nobile* and Fungus AR-18 were much different on the band pattern. The AR-18 DNA was completely digested by the three endonuclases, but the *D. nobile* DNA using other two endonucleases was incompletely digested except EcoR Ⅰ. The digested products were on hybridization with high purity band dd-1 and dd-8 as probes, respectively (Figure 3B, C). The hybridization results showed that the probes of band dd-1 and dd-8 had no hybridization signal with Fungus AR-18 genome DNA cut by the three endonucleases, but there were signals with the two enzyme (BamH Ⅰ and EcoR Ⅰ) cutting products of *D. nobile* genome DNA. The Southern blot analysis demonstrated the two differential bands were all from the genome DNA of *D. nobile* and were possible single copy because there was one hybridization band in the cut products respectively by BamH Ⅰ and EcoR Ⅰ.

The differentially expressed cDNA fragments were cloned into pBluescript Ⅱ SK (+/-) T vector and sequenced using F Primer (M13-47). The sequences of dd-1 and dd-8 were submitted to the NCBI databank with the accession numbers GR410230 and GR410231, respectively. The nucleotide sequences obtained were compared against NCBI nucleotide sequence database to identify putative proteins that are encoded by these mRNAs. We could not find a complete reading frame to known sequences in GenBank.

## DISCUSSION

Here we report the isolation of two differentially expressed

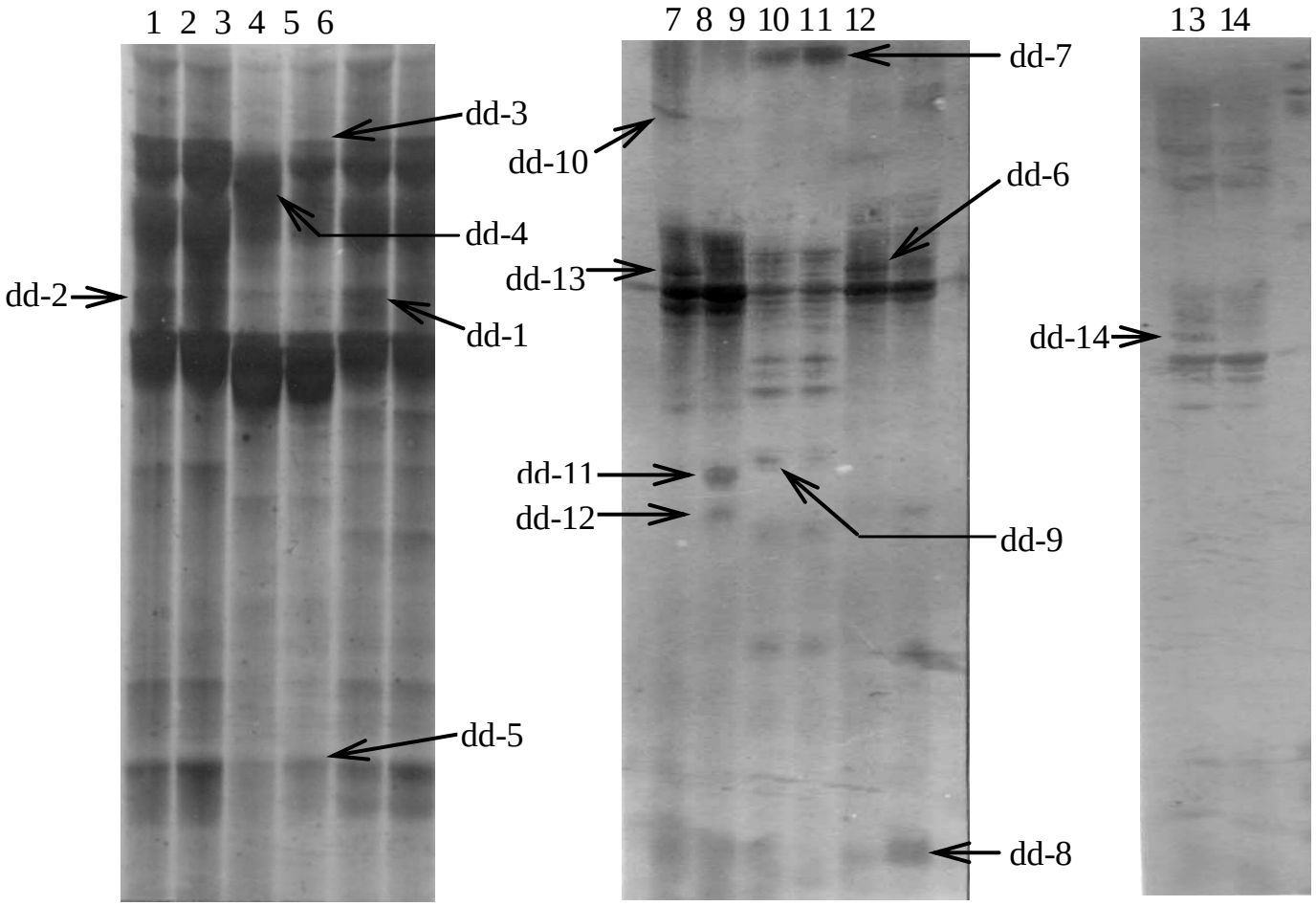


**Figure 1.** DDRT-PCR analysis of *D. nobile* induced by *Epulorhiza* sp. AR-18 promoting the growth. Total RNA extracted from the control groups and the fungus symbiotic groups were reverse transcribed and amplified with the 5'-arbitrary primer and the 3'-anchored primer (5'-AAGCTTTTTTTTTTG-3'). Amplified cDNA fragments were separated in a 6% denaturing polyacrylammide gel. Arrows indicate differentially expressed cDNA fragments that were recovered from gel and analyzed further. Inoculated the mycorrhizal fungus: Lanes 1, 3, 5, 7, 9, 11 and 13; control without inoculating the fungus: lanes 2, 4, 6, 8, 10, 12 and 14; the 5'-arbitrary primer used as lane teams, 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11 and 12, 13 and 14 were B0321, B0320, B0303, B0306, B0304, B0318, and B0311, respectively.

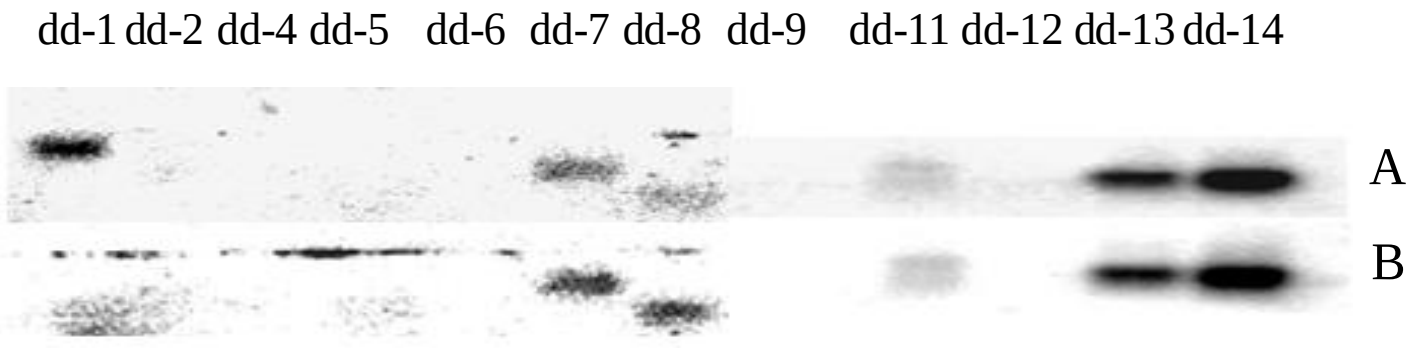


**Figure 2.** Reverse Northern blot of differentially expressed genes. Differential display cDNA bands recycled were re-amplified. Aliquot of the re-amplified products were isolated on 1.2% agarose gel and transferred on two membranes as described in Materials and methods. The membranes were hybridized with α-$^{32}$P-dCTP-labeled cDNAs that were synthesized from total RNA prepared from *D. nobile* leaves symbiotic with *Epulorhiza* sp. AR-18 (A panel) and control without fungus inducing (B panel). Lane dd-1, dd-2 ..., dd-13, and dd-14 were re-amplified cDNA bands, respectively.

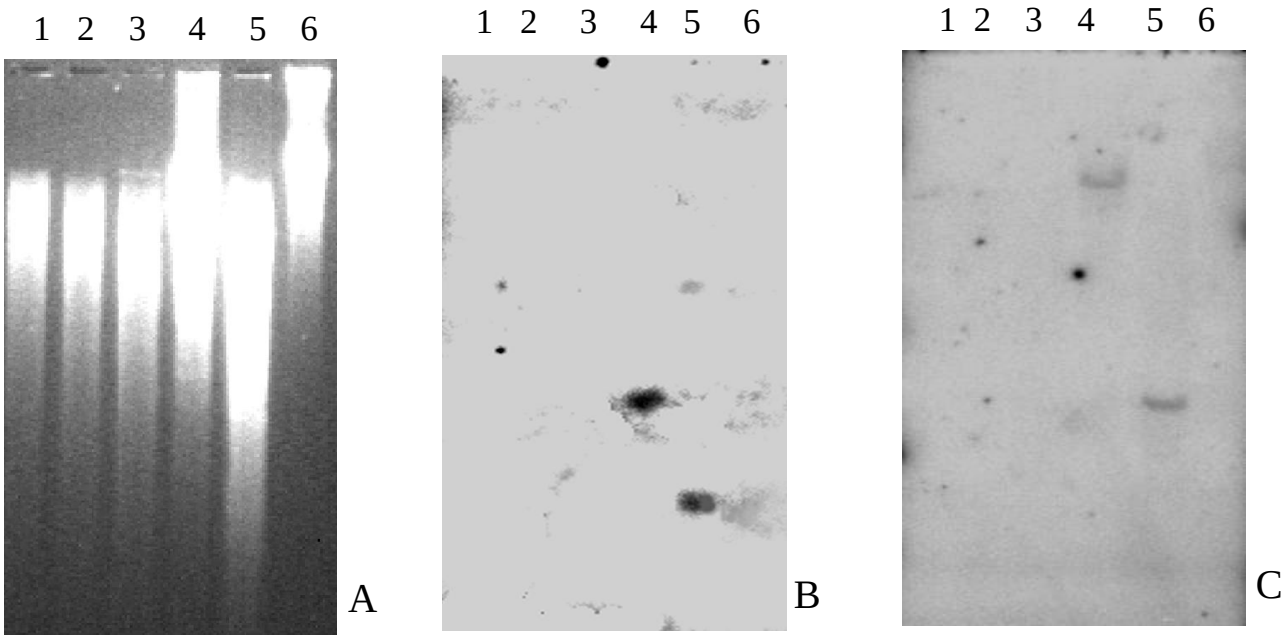


**Figure 3.** Southern blot analyses of two differential cDNA bands dd-1 and dd-8. **A** The single endonuclease cutting products of genomes of fungus AR-18 and *D. nobile* were isolated on 0.8% agarose gel and transferred on two membranes as described. Lanes 1-3: The products cut by BamH I, EcoR I and Pst I, respectively, of *Epulorhiza* sp. AR-18 genome; lanes 4 to 6: the products cut by BamH I, EcoR I and Pst I, respectively, of *D. nobile* genome. The membranes were hybridized respectively with α-$^{32}$P-dCTP-labeled dd-1 and dd-8 probes by PCR. **B** Southern blot analysis used band dd-1 as probe. **C** Southern blot analysis used band dd-8 as probe. Lanes 1-6 of B and C was same as that of A.

cDNA fragments from *D. nobile* whose growth was promoted under being symbiotic with entophytic fungi, *Epulorhiza* sp. AR-18, by applying the differential display method (Liang and Pardee, 1992; Park et al., 2010). The procedure has demonstrated its utility for the analysis of differential gene expression in very different organisms including plants (Venkatachalam et al., 2009; Xu et al., 2007) and fungi (Nagee et al., 2008). Analysis of a more complex system, like a plant-endofungi interaction, has been attempted but focusing on the study of entophytic fungi promoting plant growth (Sherameti et al., 2005; Barazani et al., 2007; Vadassery et al., 2009). To our knowledge this is the first report on DDRT-PCR analysis of the interaction between the plant growth-promoting entophytic fungi and Dendrobium.

In our study, we focused on the differentially expressed genes of plant whose growth was enhanced induced by the entophytic fungus. Originally, it would be the best to collect the roots of plant being symbiotic with the fungus, but the genomes of fungus and plant in the roots were not easy to be isolated. So we cut off the roots only retained the aerial parts as test materials. Thus it caused some differentially expressed genes in the roots lost. However, our previous works showed that the effect of *Epulorhiza* sp. AR-18 on fresh weight had significant difference ($P<0.05$) in vermiculite medium. Fresh weight of *D. nobile* inoculated with *Epulorhiza* sp. AR-18 increased by 16% compared with the non-inoculated ones (control). The effect of *Epulorhiza* sp. AR-18 on dry weight of *D. nobile* had very significant difference ($P<0.01$). Dry weight of *D. nobile* inoculated with *Epulorhiza* sp. AR-18 was raised by 21% compared with the control (Song and Guo, 2001). It suggested that the genes expression related to growth on aerial parts of *D. nobile* were enhanced by inoculating with *Epulorhiza* sp. AR-18. Therefore, it was available to select the stems and leaves as experimental materials.

There is controversy about the sensitivity of DDRT-PCR procedure. In the present work, the differential bands used to hybridization were in total 12, of which true differential bands were only 2 (dd-1 and dd-8). The false positive rate was 83%, which was high and similar to the results of other reports (Bertioli et al., 1995; Unver et al., 2008). Thereby, it was very important using Northern blot or reverse Northern blot to identify the differential bands facticity. In contrast to Northern blot, the detection efficiency of reverse Northern blot is higher. For reverse Northern blot, over 10 true or false cDNA bands could be detected by one time hybridization. Moreover, its operation need not strictly prevent RNA degradation as Northern blot but as Southern blot. However, this method might bring about some truly differential bands lost, such as the band dd-2, dd-4, dd-5, dd-7, dd-9 and dd-13 in this research, of which were possible truly positive bands.

The main reason might be that labeling cDNAs in the course of reverse Northern blot were by means of reverse transcription reaction, in which leaking labels were occurred possibly for low even for high abundance transcripts. But Northern blot might detect the low abundance transcripts via ascending the concentration of probes. Hence, combination with the advantages of two blot methods would be used in practical work (Ludwig-Müller et al., 2005).

Southern blots were applied to identify the two differential cDNAs bands (dd-1 and dd-8) from the genome of *D. nobile* or *Epulorhiza* sp. AR-18. The results provided a proof to further study the full-length and function of the differential genes. But the sequence analysis and database searches revealed no significant homology of these fragments to any known sequences, neither at nucleotide nor at amino acid level. Although the sequence data do not provide relevant information about the nature of the isolated fragments, they can be used as probes to isolate the full-length cDNA and genomic copies. Our study presents a preliminary analysis attempting to find putative genes playing roles in the promoting-growth mechanism of *D. nobile* induced by *Epulorhiza* sp. AR-18. Further analysis should be undertaken to find more candidate genes on the samples of roots and those in different symbiotic ages.

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