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Screening for differentially expressed genes in *Anoectochilus roxburghii* (Orchidaceae) during symbiosis with the mycorrhizal fungus *Epulorhiza* sp.

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Mycorrhizal fungi promote the growth and development of plants, including medicinal plants. The mechanisms by which this growth promotion occurs are of theoretical interest and practical importance to agriculture. Here, an endophytic fungus (AR-18) was isolated from roots of the orchid *Anoectochilus roxburghii* growing in the wild, and identified as *Epulorhiza* sp. Tissue-cultured seedlings of *A. roxburghii* were inoculated with AR-18 and co-cultured for 60 d. Endotrophic mycorrhiza formed and the growth of *A. roxburghii* was markedly promoted by the fungus. To identify genes in *A. roxburghii* that were differentially expressed during the symbiosis with AR-18, we used the differential display reverse transcription polymerase chain reaction (DDRT-PCR) method to compare the transcriptomes between seedlings inoculated with the fungus and control seedlings. We amplified 52 DDRT-PCR bands using 15 primer combinations of three anchor primers and five arbitrary primers, and nine bands were re-amplified by double primers. Reverse Northern blot analyses were used to further screen the bands. Five clones were up-regulated in the symbiotic interaction, including genes encoding a uracil phosphoribosyltransferase (UPRTs; EC 2.4.2.9) and a hypothetical protein. One gene encoding an amino acid transmembrane transporter was down-regulated, and one gene encoding a tRNA-Lys (trnK) and a maturase K (matK) pseudogene were expressed only in the inoculated seedlings. The possible roles of the above genes, especially the UPRTs and matK genes, are discussed in relation to the fungal interaction. This study is the first of its type in *A. roxburghii*.

***Anoectochilus roxburghii*, orchid mycorrhizal symbiosis, *Epulorhiza* sp., differential display-PCR (DD-PCR), gene screening**

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Anoectochilus roxburghii (Orchidaceae) is a phanerophyte that grows in humus soils in misty and humid broad-leaved forests [1]. This plant was originally used as a source of the precious natural drugs known as 'King Medicine' in China. Extracts from this plant have diverse pharmacological effects, such as liver protection, cancer prevention, antioxidant activities, and anti-inflammatory effects [2]. It has been widely used as a folk medicine to treat cancers and cardiovascular, lung, and liver diseases, and it is given to underdeveloped children in China and other Asian countries [3].

A. roxburghii is now facing extinction because of destruction of its habitat, combined with heavy exploitation of its wild resources, its low propagation rate, and its slow growth [4]. All members of the Orchidaceae are symbiotic with fungi under field conditions [5,6]. In the field, wild *A. roxburghii* commonly forms mycorrhizal symbiotic relationships with some endophytic fungi. Therefore, screening

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for beneficial fungi that promote the growth and development of *A. roxburghii*, and studying the plant-fungus interaction is of great theoretical significance, and will help to preserve this germplasm resource through the development of new progenitive approaches [7].

In our previous work, an endophytic fungus, AR-18, a species of *Epulorhiza*, was successfully isolated from the roots of wild-grown *A. roxburghii* [8]. When seedlings of *A. roxburghii* were inoculated with *Epulorhiza* sp. AR-18 *in vitro*, they showed a promotion in growth, increased survival rate and yield, increased activities of four enzymes [9], a greater number of buds and roots per plant [10], and increased contents of polysaccharides and flavones [5,11]. These results indicate that some genes in *A. roxburghii* are differentially expressed in plant-fungus symbioses. In this study, we searched for genes with altered expression in the presence of the endophytic fungus.

To identify and isolate differentially expressed genes, several techniques based on the polymerase chain reaction (PCR) can be used [12,13]. Differential display reverse transcription (DDRT)-PCR can identify novel transcripts by comparative analysis of more than two samples. Therefore, integrating DDRT-PCR and microarray analysis should provide a more comprehensive approach to understand the molecular mechanisms of the symbiosis between medicinal plants and fungi [14,15]. Furthermore, DDRT-PCR has several advantages over other methods: the techniques are fast, simple, and widely accessible; it sensitively detects low-abundance mRNA transcripts and requires only small amounts of RNA; both induced and repressed genes can be detected simultaneously, and more than two samples can be compared [16]. The objective of this study was to identify genes that are differentially expressed in *A. roxburghii* during its mycorrhizal symbiosis using a differential display technique [14,17,18]. Comparative molecular studies will provide information that will increase our understanding of the mechanism by which an endophytic fungus improves the growth and development of *A. roxburghii*.

1 Materials and methods

1.1 Plant materials

Seedlings from the same clone of *A. roxburghii* were grown on improved MS medium [19], which was supplemented with 10% (w/v) active carbon, 3% (w/v) sucrose, and 0.8% (w/v) agar, pH 5.8. Seedlings were grown in a conventional greenhouse with a 12-h light/12-h dark photoperiod at (24±1)°C, and an illumination intensity of 2000 Lx.

1.2 Culturing and identification of the endophytic fungus

The representative strain (No. AR-18) was stored at low temperatures (0–4°C) at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College. The strain was inoculated on a plate of wheat bran medium, consisting of 30 g dm^{-3} wheat bran, 1.5 g dm^{-3} $MgSO_4$, 3 g dm^{-3} KH_2PO_4, 20 g dm^{-3} D-glucose, and 14 g dm^{-3} agar. Before inoculation, the fungus was activated by culturing for 10–15 d at (24±1)°C in the dark. Discs were excised from the medium using a hole puncher (Φ11 mm), and were then used as the inocula. The mycelia of the fungal strain were used to prepare water squash slides, which were observed under a light microscope. The fungus was identified as a *Epulorhiza* species based on the outline of the colonies, formation of concentric rings and aerial hyphae, color of the aerial hyphae, color of the colony centre and its reverse side, thickness and texture of the colony, and the shape and dimension of monilioid cells [20,21].

1.3 Inoculation of fungi onto *A. roxburghii* roots and their symbiotic structure

The tissue-cultured seedlings of *A. roxburghii* transplanted on MS medium for 10 d were co-cultured with the activated *Epulorhiza* sp. AR-18 at 25°C. After the seedlings were co-cultured for 60 d, they were harvested and used as the inoculated group. Seedlings grown in the same conditions but without endophytic fungus were used as the control group [22]. When the seedlings were collected, all of the roots were removed, and the remaining tissue was frozen immediately at –80°C.

Roots of *A. roxburghii* co-cultured with *Epulorhiza* sp. AR-18 for 60 d were cut into 3–7 mm pieces and fixed immediately in formaldehyde-acetic acid-50% alcohol, dehydrated, embedded in paraffin, and sectioned with a microtome. Sections were mounted on slides and heated gently in a water bath and stained with safranin O/fast green (Sigma Chemical Co.) for 10 min. Slides were then rinsed with distilled water and allowed to dry [23]. Stained tissues were viewed and photographed directly using a digital camera.

1.4 RNA isolation and purification

Total RNA was isolated from 1 g stem and leaf tissues from the treated and control group using the improved CTAB method as described by Chang *et al.* [24]. The RNA yield and quality were measured by determining absorbance at 230, 260, and 280 nm with a Hitachi 2000 spectrophotometer. RNA quality was checked by RNA gel analysis using a horizontal 1.0% agarose gel. The gel was visualized and photographed under ultraviolet light after staining with ethidium bromide. The RNA pellets were stored in ethanol at –80 °C until use. Total RNA (20 μg) was treated with 40 U RNaseOUT™ (Invitrogen, USA) and 1 U DNase (Invitrogen) in a solution of 20 mmol L^{-1} Tris-HCl (pH 8.4), 2 mmol L^{-1} $MgCl_2$, and 50 mmol L^{-1} KCl at room tempera-

ture for 15 min. Then, 1 μL EDTA (25 mmol L^{-1}) was added and the mixture was incubated at 65°C for 10 min to end the reaction.

1.5 First strand cDNA synthesis and DDRT-PCR

Complementary DNA was synthesized for 10 min at room temperature in a 20-μL aqueous reaction mixture containing 5.5 μL DEPC-treated water, 5 μL (2 μg) DNA-free total RNA, 4 μL 5× first strand buffer (Gibco-BRL, Eggenstein, Germany), 0.5 μL of 10 mmol L^{-1} of each dNTP, 2 μL 0.1 M DTT (Gibco-BRL), 2 μL 20 ng μL^{-1} anchor primer designated AAGC(dT_{10})-M (where M is A, G or C; Sangon, Shanghai, China), 1 μL 30 U μL^{-1} RNasin (Sangon) and 1 μL 200 U μL^{-1} M-MLV reverse transcriptase (Gibco-BRL). Then the mixture was incubated at 42°C for 50 min and heated to 48°C for 30 min. Following the reaction, the cDNA was incubated at 75°C for 10 min to inactivate the reverse transcriptase, and then stored at −20°C until use.

The polymerase chain reaction (PCR) mixture consisted of 2 μL cDNA from the reverse transcription (RT) reaction, 0.5 μL dNTP (10 mmol L^{-1} of each), 2 μL 10× PCR buffer, 1.2 μL 25 mmol L^{-1} $MgCl_2$, 2 μL arbitrary primer (20 ng μL^{-1}), 2 μL anchor primer H-T_{10}M (5′-AAGCTTTTT-TTTTTM-3′, M=A, G or C) (20 ng μL^{-1}) and 0.4 μL Taq polymerase (Sangon) completed to a volume of 20-μL with DEPC-treated ddH_2O. The PCR conditions were as follows: 25 cycles of 94°C for 30 s, 40°C for 2 min, 72°C for 1 min, followed by 10 cycles of 94°C for 30 s, 50°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. Prior to PCR, the mixture was heated to 94°C for 5 min. Five random primers (DD10, 5′TGCTCCCGGCCGCCAAGC3′; DD20, 5′TGCCGGAATTCTGGTCAT3′; DD23, 5′TGCT-CCCGGCCGCCATGG3′; DD34, 5′TGCCGGAATTC-TGGTGAC3′; DD60, 5′TGCCGGAATTCCGA- CTGT3′; Sangon) were chosen. A total of 15 primer combinations were screened.

1.6 Gel electrophoresis and re-amplification

DDRT-PCR samples were mixed with loading dye (formamide, bromphenol blue, xylene cyanol), denatured at 95°C for 10 min, cooled on ice, and loaded onto 6% polyacrylamide denaturing gels. Gels were run at 60 W for 4–6 h, and PCR products were detected by silver staining [25–27].

Using a sharp, clean razor blade, the fragments determined to be differentially displayed bands were excised from the gels and soaked in 20 μL ddH_2O at room temperature for at least 10 min and then in a water bath at 90–100°C for 20 min. After a short centrifugation, the liquid was transferred to a clean Eppendorf tube. The extracted DNA was used directly as the template for PCR [18]. A 4-μL aliquot of the extracted DNA solution was used in a 40-μL reaction mixture to reamplify the excised cDNA band. The constituents of the PCR mixtures were identical to those of DD-PCR except for the template. Re-amplification reactions were as follows: 94°C for 5 min, then 30 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. After reamplification, a 10 μL aliquot of the PCR reaction was mixed with 2 μL of gel loading dye and separated by electrophoresis on a 1.2% agarose gel with ethidium bromide to ensure that a fragment of the correct size was amplified.

1.7 Reverse Northern blot analysis

A 20-μL aliquot of the reamplified products was dotted on each Hybond-N^+ membrane filter (Amersham Pharmacia, Piscataway, NJ, USA) and used for reverse Northern blotting. The products of reverse transcription were used as probes. The probes were prepared as follows: 10 μg RNA treated by DNase I was mixed with 2 μL 20 μmol L^{-1} anchor primer oligo-dT_{18} and the mixture denatured at 65°C for 5 min, rapidly cooled on ice for 2 min, and then added to tubes containing 1 μL 40 U RNaseOUT (Invitrogen), 6 μL 5× First-Strand buffer, 3 μL 0.1 mol L^{-1} DTT, 5 μL DIG DNA Labeling Mix (Roche, Mannheim, Germany) and 1 μL Superscript II. The reaction mixture was incubated at 42°C for 60 min, then at 48°C for 30 min, and finally at 70°C for 10 min to end the reaction. Hybridization and labeling of the cDNA probes were performed as described in the manual of the DIG High Prime DNA Labeling Detection Starter Kit I (Roche).

1.8 Cloning of differentially displayed cDNAs

The cDNA fragments confirmed by reverse Northern blot analysis were amplified. The PCR products were extracted from a 0.8% agarose gel with the DNA Gel Extraction Kit (Yuanpinghao Biotech Co. Ltd., Beijing, China). The extracted cDNA fragment was inserted into a *pGEM-T* vector (Promega, USA) according to the manufacturer's instructions and cloned into *Escherichia coli*. Plasmid DNA with the insert was extracted from *E. coli* using the alkaline lysis method [28]. An aliquot (1–2 μL) of the extracted plasmid DNA was cut with 0.5 μL of the restriction enzyme *Eco*R I at 50°C for 2–4 h and then incubated at room temperature overnight. The insert was visualized by 1.4% agarose gel electrophoresis or PCR amplification using the same primer combinations and PCR conditions.

1.9 DNA sequencing and similarity analysis

Plasmids were isolated from randomly selected clones using the QIAprep Spin Miniprep kit (Qiagen, Germany). Plasmids were sequenced by the Shanghai Bioasia Biotechnology Co. BLAST searches against sequences in the GenBank databases were conducted to identify DNA sequence homologs.

2 Results

When *Epulorhiza* sp. AR-18 and the tissue-cultured seedlings of *A. roxburghii* were co-cultured for 2–3 weeks, the mycelia started to make contact with the roots, whose surfaces gradually changed from a green to a tawny color (Figure 1A). The association with *Epulorhiza* sp. AR-18 clearly promoted the growth of *A. roxburghii*, as seedlings grown with the fungus for 35 d showed an average growth increase of 10.3% compared with control seedlings (data not shown).

Epulorhiza sp. was identified from its morphological characteristics such as colony color, and the shape and dimensions of monilioid cells [29]. On PDA, fungal colonies were white and grew rapidly, reaching 7 cm in diameter after 5 d incubation at 25°C. The hypha were arranged closely, and white aerial hyphae seldom developed. Some of the cultures showed inconspicuous concentric rings (Figure 1B). The hyphae were hyaline, 2–7.5 mm in diameter, and superficial with a submerged margin. Monilioid cells were barrel-shaped to cylindrical (Figure 1C). These morphological characteristics indicated that the endophytic fungus belonged to the genus *Epulorhiza*.

The structure of the mychorriza that formed when *Epulorhiza* sp. AR-18 and *A. roxburghii* were co-cultured for 60 d was examined under a light microscope, and its characteristics indicated that it was among the typical mycorrhizae of orchids [30]. The hyphae invaded into the outermost 3–4 layers of cells in the cortical tissue beneath the epidermis. The hyphae consisted of pelotons and mycelial branches in the outer cortical cells, and pelotons and mycelial splinters in the inner cortical cells (Figure 1D). Compared with normal cells, some of the cortical cells showed plasmolysis or an enlarged nucleus.

DDRT-PCR experiments were carried out using a total of 15 DD primer combinations, using tissue samples from plants incubated for 60 d with the fungus and from the control. After analysis of silver-stained images, 52 differentially expressed bands were selected and excised from the sequencing gel (Figure 2). The selection was based primarily on the presence or absence of a band or on differences in intensity of a particular band between the treated and control samples.

To reamplify the gel-extracted bands, the PCR reaction conditions were altered, and the bands that could be amplified by double primers (anchor primer and random primer) were selected for further study. We did not conduct further analyses on excised bands that could only be amplified by a single anchor primer or a random primer. In total, nine bands that were able to be reamplified by double primers (Figure 3) were excised from the agarose gel, and the DNA

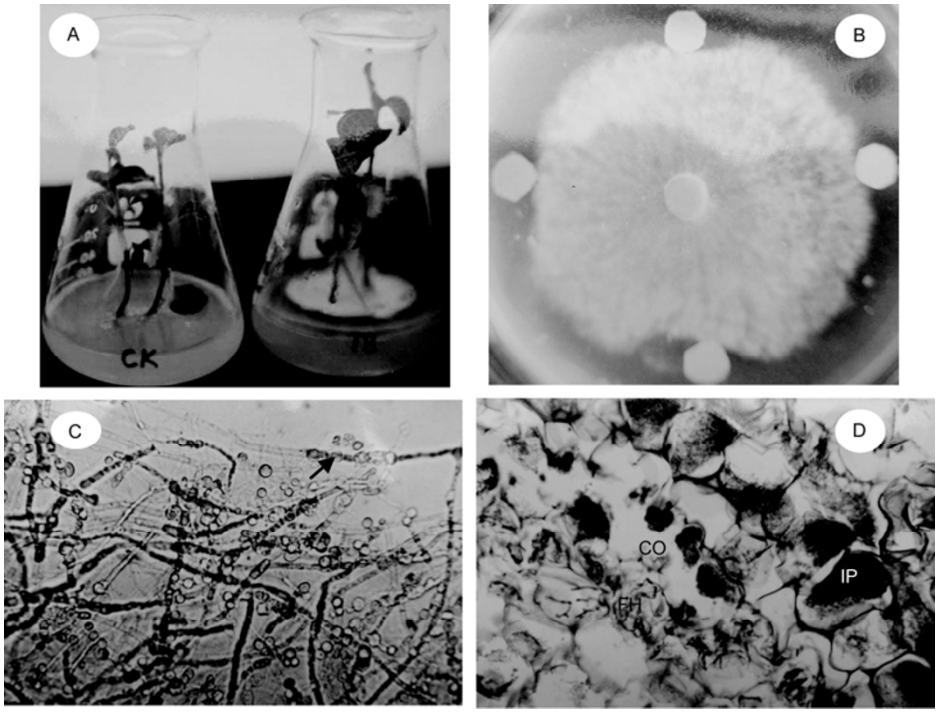

Figure 1 Growth state and structure formed by mycorrhizal *Epulorhiza* sp. AR-18 associated with tissue-cultured seedlings of *Anoectochilus roxburghii* co-cultured for 60 d. A, Tissue cultured seedlings inoculated with *Epulorhiza* sp. AR-18, or uninoculated (control). B, Colony of *Epulorhiza* sp. AR-18 on PDA after 5 d at 25°C. C, Extraradical mycelia of *Epulorhiza* sp. AR-18 extracted from the mycorrhizosphere of *A. roxburghii* after 60 d co-culture. Monilioid cells with slight tubular constriction (arrowhead) (×224). D, Microstructure of symbiosis of *A. roxburghii* and *Epulorhiza* sp. AR-18 (×448). IP, intact peloton; CO, cortex cell; FH, fungus hypha.

Figure 2 Some differential display band patterns of mRNA from control and fungus-inoculated *A. roxburghii*. Total RNA extracted from the control (uninoculated) and fungus-inoculated *A. roxburghii* tissue cultured seedlings was reverse-transcribed and amplified with a 5′-arbitrary primer and a 3′-anchored primer. Amplified cDNA fragments were separated on a 6% denaturing polyacrylamide gel. Arrows indicate differentially expressed cDNA fragments that were recovered from the gel and further analyzed. Products from the control are in lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 and 23. Products from the fungus-inoculated seedlings are in lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24. Primer combinations of lanes 1 and 2, 3 and 4,…, 23 and 24 were the same between the two groups. Primer combinations used in lanes 1–8 were H-T_{10}A and DD10, DD20, DD23, DD34, respectively; lanes 9–16 were H-T_{10}G and DD10, DD20, DD23, DD34, respectively; lanes 17–24 were H-T_{10}C and DD10, DD20, DD23, DD34, respectively. M, DNA marker (DL2000).

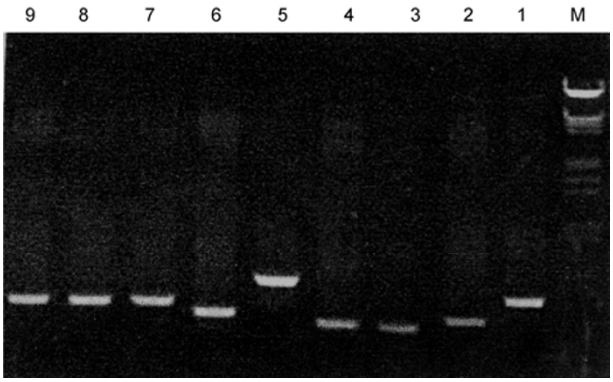

Figure 3 Only the fragments reamplified by double primers of differentially expressed bands were extracted. Lanes 1–9 show differentially expressed bands: AR-DD17, AR-DD19, AR-DD20, AR-DD23, AR-DD44, AR-37, AR-DD47, AR-DD38 and AR-DD40, respectively. M, DNA marker (DL2000).

was extracted for use in reverse Northern blot analysis as further confirmation. Seven fragments were confirmed in the reverse Northern blot analysis: AR-DD017, AR-DD019, AR-DD020, AR-DD023, AR-DD037, AR-DD044, and AR-DD047 (Figure 4).

Of those, the genes AR-DD017, AR-DD019, AR-DD037, AR-DD044, and AR-DD047 were up-regulated in *A. roxburghii* after inoculation with the fungus AR-18. AR-DD020 was down-regulated, and AR-DD023 was expressed only in the treated group. The seven fragments representing differentially expressed genes were cloned into the pGEM-T vector. The clones of the seven fragments were amplified by corresponding primer combinations. Plasmid DNA was extracted from the clones that were confirmed to have target inserts by restriction enzyme digestion.

The above-mentioned seven clones were sequenced, and

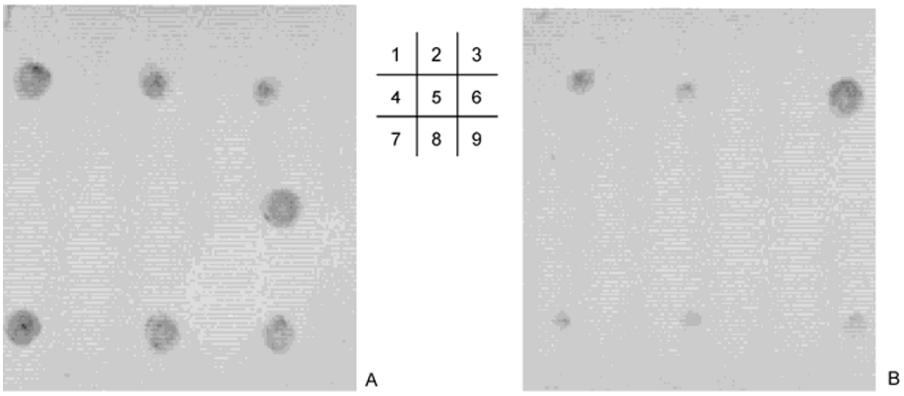

Figure 4 Reverse Northern dot blot of differentially expressed genes. Extracted cDNA bands were reamplified, and aliquots of amplified products were blotted on two membranes as described in Materials and methods. Membranes were hybridized with DIG-labeled cDNAs that were synthesized from total RNA prepared from control (B) and fungus-inoculated *A. roxburghii* leaves (A). Dots 1–9 are AR-DD17, AR-DD19, AR-DD20, AR-DD38, AR-DD40, AR-23, AR-DD37, AR-DD44, and AR-DD47, respectively.

the sequences were submitted to NCBI databank under the accession numbers shown in Table 1. The putative functions of the genes were predicted by sequence similarity to genes or proteins of known function in the NCBI database. However, three of them, AR-DD037, AR-DD044 and AR-DD047, showed no significant similarity to sequences in GenBank and are potentially unique genes. Of the other two up-regulated genes, AR-DD019 showed high homology to uracil phosphoribosyltransferase of *Ricinus communis* (81% identity), and AR-DD017 showed low sequence identity (46%) with a hypothetical protein of *Gibberella zeae*, a species of fungus. A portion of the cDNA of clone AR-020 showed 100% homology to a partial sequence of an amino acid transmembrane transporter of *A. thaliana.* The clone AR-DD023, which was expressed only in fungus-inoculated seedlings, showed 96% identity with the tRNA-Lys (trnK) gene and the maturase K (matK) pseudogene of *A. roxburghii*.

3 Discussion

The macro-morphology and microstructure of *A. roxburghii* and *Epulorhiza* sp. AR-18 indicated that the plant and the fungus formed an endomycorrhiza in the co-culture system in these experiments. Our observations are similar to those reported by Hadley *et al.* [31] for *Spathoglottis plicata* Bl. We searched for genes with altered expression in the presence of the endophytic fungus, and 7 cDNA fragments were screened from 52 bands. Among the cDNAs isolated, five genes were up-regulated, one gene was down-regulated and one gene was specifically expressed in *A. roxburghii* inoculated with *Epulorhiza* sp. AR-18. After comparison of these sequences with those in data banks, we identified four genes with low or high homology to known genes.

The first fragment (AR-DD017) identified in our analysis is a hypothetical protein. The homologs of AR-DD017 identified via BLASTN and BLASTX alignments were not homologous with any known plant proteins, but instead were partially identical to the predicted or hypothetical proteins of some fungi and animals, including *G. zeae*, *Photorhabdus asymbiotica*, *Nematostella vectensis*, and *Taeniopygia guttata*. Therefore, the exact function of this protein in fungus-promoted plant growth is uncertain.

The second fragment (AR-DD019) identified in our analysis was up-regulated in *A. roxburghii* inoculated with AR-18: it encodes a uracil phosphoribosyltransferase, for which there are homologs in many plants, including *Ricinus communis*, *Nicotiana tabacum*, *Zea mays*, and *Arabidopsis thaliana*. Uracil phosphoribosyltransferases (UPRTs; EC 2.4.2.9) are involved salvaging pyrimidines by catalyzing the formation of uridine monophosphate (UMP) from uracil and phosphoribosylpyrophosphate [32]. UPRTs have been described as non-essential, energy-saving enzymes. However, it was reported recently that uracil salvage is of major importance for plant development [33]. The inability to salvage uracil caused a light-dependent dramatic pale-green to albino phenotype, dwarfism, and the inability to produce viable progeny in loss-of-function mutants. Plastid biogenesis and starch accumulation were affected in all analyzed tissues, with the exception of stomata [33]. A single nuclear gene, *UPP*, encodes the protein, which is targeted to plastids and accounts for almost all UPRT activity in *Arabidopsis* [33,34]. Previous research found that some endophytic fungi isolated from the roots of orchids had a remarkable growth-promoting effect [5,10]. In the present work, the expression of a gene encoding UPRT of *A. roxburghii* was enhanced after co-culturing with AR-18. This illustrates that the capacity for uracil salvage was increased and plastid biogenesis was effectively promoted in *A. roxburghii*. These data suggest that the endophytic fungus may promote the growth and development of *A. roxburghii* via the enhancement of *UPRT* gene expression.

The other fragment (AR-DD020) identified in our analy-

Table 1 Sequence homologies of differentially expressed *A. roxburghii* cDNA fragments found in NCBI sequence database

DD-clones	Bp	GenBank EST-accessions	BLAST hits	GenBank accessions of hits	BLAST scores	Positive scores	
						(aa)	(%)
AR-017	410	GR410423	Hypothetical protein (*Gibberella zeae*)	XP_391017	7.5	15/32	46
AR-019	347	GR410424	Uracil phosphoribosyl-transferase (*Ricinus communis*)	EEF32516.1	8×10^{-23}	99/122	81
AR-020	307	GR410425	Amino acid transmembrane transporter (*Arabidopsis thaliana*)	NP_177862.1	0.074	15/15	100
AR-023	311	GR410426	tRNA-Lys (trnK) gene and maturase K (matK) pseudogene, (*Anoectochilus roxburghii*)	EU817409	2×10^{-124}	88/92	96

sis was down-regulated by AR-18. This gene has nucleic acid sequence similarity to the amino acid transmembrane transporter of *A. thaliana*. In plants, amino acids play fundamental roles in a multitude of processes, including protein synthesis, hormone metabolism, cell growth, production of metabolic energy, nucleotide synthesis, nitrogen metabolism, and urea biosynthesis [35]. In multicellular organisms, many nitrogenous compounds are transported between cells. As mediators of this process, amino acid transporters have been physiologically characterized in plants. Many studies suggest that there are multiple transporters that differ in their substrate spectrum, transport mechanism (i.e., ions used in cotransport), and tissue specificity [36–38]. The nitrogen metabolic pathway is very complicated: some of the reactions are catalyzed by multiple enzymes in different cellular locations, and the known amino acid transporters can be grouped into five superfamilies [35,39]. Therefore, the reduced expression of an amino acid transmembrane transporter in the presence of *Epulorhiza* sp. AR-18 suggests that it may be a small part of a global mechanism by which the endophytic fungus *Epulorhiza* sp. AR-18 controls the transcriptional state of a set of genes.

The fourth fragment (AR-DD023) identified in our analysis was expressed only in seedlings inoculated by *Epulorhiza* sp. AR-18: sequence alignment predicted that it encodes a putative maturase K (matK) protein. This enzyme is encoded by $tRNA^{Lys}$ introns in higher plant chloroplasts and by freestanding open reading frames in the residual plastid genomes of non-photosynthetic plants. The function of the enzyme is in the splicing of multiple group II introns [40]. These nuclear maturase-like proteins may be imported into organelles to function in group II intron splicing and/or they may have other cellular functions. Nuclear-encoded maturases could regulate organellar gene expression and may reflect a step in the evolution of mobile group II introns into spliceosomal introns [40,41]. Because maturase-encoding group II introns are present in plant mitochondria and chloroplast genomes, it is likely that the open reading frames of nuclear maturases were transferred from an organelle to the nucleus, as has been documented for other organellar genes [42,43]. Thus, the nuclear-encoded maturase could potentially function as part of a common splicing apparatus for multiple organelle group II introns. The transfer of group II intron maturases to the nucleus may have a role in regulation of organellar gene expression; that is, it may link the splicing of one or more organellar introns to global signals that regulate gene expression in response to the cellular energy state or environmental stimuli [44]. The fact that the growth and development of a plant is improved by a symbiosis with an endophytic fungus suggests that fungus-induced changes in plant growth may involve interactions between fungal movement proteins and plant proteins.

In conclusion, we surveyed putative genes playing roles in the growth promotion of *A. roxburghii* during its symbiotic relationship with the endophytic fungus, *Epulorhiza* sp. AR-18. We identified several novel fungus-responsive genes. Further studies are required to confirm their functions by expressing the full-length sequence of each gene. The full-length sequences of these fragments will provide valuable data for the identification of the complete *A. roxburghii* transcriptome. Also, they will provide new molecular resources to study the growth promotion and symbiotic relationships of the endophytic fungus with *A. roxburghii*. These results also showed that the differential display technique is a useful tool for analyzing the molecular mechanism by which endophytic fungi promote growth of the Orchidaceae.

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