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ORIGINAL ARTICLE

Screening of taxol biosynthesis-related genes in taxol produced from *Nodulisporium sylviforme* HDF-68 by mRNA differential display

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Abstract Differential expressed genes in taxol producing *Nodulisporium sylviforme* HDF-68 at different periods of fermentation were analyzed by mRNA differential display. A total of 38 fragments were found expressed differentially during taxol synthesis phase. Using reverse Northern blot analysis, the hybridization signals of 16 fragments were consistent with the results of differential-display reverse transcription-PCR (DDRT-PCR). Sequencing results showed that nucleic acid sequences of TBR3, TBR4 and TBR7 were extremely similar to those of human, and the genes of cytochrome oxidase subunit I in TBR11 and *Neurospora crassa* mitochondrial were similar. No other functions could be identified by homolog analysis of the rest of expressed sequence tag (EST) sequences. In total, 16 open reading frames (ORFs) were found in these 16 ESTs, and seven of them have homologous proteins in the NCBI protein database. However, only the functions of TBR10 and TBR11 homolog were known; others were hypothetical proteins. Furthermore, the results also showed that the limitation of information about amplification product in 3′-UTR region could be overcome through a single random primer, instead of the anchor primer to combine with random primer in DDTR-PCR. This study provides valuable information for identifying functionally related genes in taxol-producing endophytic fungi and clues on further defining the taxol biosynthetic pathway.

Keywords Endophytic fungi · Taxol · Biosynthesis · cDNA cloning · mRNA differential display

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Introduction

Taxol is a diterpenoid with anticancer activities. It was first isolated from the bark of *Taxus brevifolia* (Wani et al. 1971). It is widely used in the treatment of a variety of cancer diseases, including carcinomas of the ovary, breast and cervix (Jones et al. 1996; Pulkkinen et al. 1996; Woo et al. 1996). At present, taxol is still mainly extracted from the bark of yews. However, this method can not meet the increasing demand for taxol on the market, because yews grow very slowly and are an endangered species.

Recently, increasing efforts have been made to develop alternative means of taxol production, such as using complete chemical synthesis, semi-synthesis and *Taxus* spp. plant cell culture. Using microbe fermentation in the production of taxol would be a prospective method for obtaining a large amount of taxol. However, a major limitation of this method is that available taxol-producing fungi have exhibited low yield, thus preventing industrial production. In addition, there has been a lack of molecular research on taxol biosynthesis in endophytic fungi (Zhao et al. 2008a). Therefore, a detailed understanding of the molecular basis and genetic regulation mechanisms of taxol biosynthesis in endophytic fungi could be the premise for development of advanced biotechnology for large-scale taxol production.

Although studies on taxol-biosynthesis pathway in *Taxus* spp. cells have made great advances in isolating the genes and enzymes involved (Hu et al. 2000; Walker and Croteau 2000a, b; Walker et al. 2000; Jennewein et al. 2001; Schoendorf et al. 2001; Walker et al. 2002a, b; Guo et al. 2004; Chau and Croteau 2004), there have been no reports about taxol-synthesis–related genes from taxol-producing fungi. Our

group has tried to amplify taxol-synthesis–related genes by polymerase chain reaction (PCR) or reverse transcription PCR (RT-PCR) according to reported taxol synthesis-related gene sequences of *Taxus* cells, but have been unsuccessful. Taxol synthesis kinetics indicate that endophytic fungi synthesize taxol by biological fermentation, which can be divided into two phases: non-taxol–synthesis phase and taxol-biosynthesis phase. The former is in the delay period of the fungal cell cycle; the latter is in the logarithmic phase and stationary phase, in which taxol production and accumulation occur. Therefore, systematically comparing gene expression differences between the taxol-biosynthesis phase and non-taxol–synthesis phase may be useful in understanding the genetic regulation. Differential display is a powerful tool for comparing gene expression differences between two or more mRNA populations (Liang and Pardee 1992). This assay possesses the following advantages: (1) it is based on well-established methods, (2) more than two samples can be compared simultaneously, and (3) only a small amount of starting material is needed (Bauer et al. 1993; Liu 2010). It has been widely used for isolating differentially expressed gene clones. In fact, a survey of National Center for Biotechnology Information using "differential display" still yielded more than 500 hits in 2010–2011 (Curtiss et al. 2012).

Based on our previous work, the aim of this study was the identification of differential expressed genes in taxol-producing fungus HDF-68 in a different period of fermentation using differential-display reverse transcription-PCR (DDRT-PCR). A total of 38 differentially expressed bands were obtained. By reverse Northern blot analysis, 22 fragments were false positive, and hybridization signals of 16 fragments were consistent with the DDRT-PCR results. The acquired fragments are all in the coding region because one or more open reading frames (ORFs) are found in all of the 16 expressed sequence tags (ESTs). In addition, seven EST products are found in protein databases. However, only homologous proteins of TBR10 and TBR11 are known; others are hypothetical proteins. The results provide new evidence and directions for studying the relationship between endophytic fungi and the host, in addition to comparing to cell cultures producing taxol.

Materials and methods

Strains

Neurospora sylviforme HDF-68, a taxol-producing endophytic fungus with a taxol output of 468.62 μg/l, and mutants $UV_{40\text{-}19}$ and $UL_{50\text{-}6}$, originated from protoplasts of strain NCEU-1 by UV and LiCl mutagenesis (Zhao et al. 2005). The taxol output of these mutants is 376.38 and 392.63 μg/l, respectively (Zhao et al. 2011). *E. coli* $DH_{5\alpha}$ was kindly provided by laboratory of molecular biology in Heilongjiang University.

Media

Potato dextrose agar (PDA) liquid medium (Shen et al. 2000), PDA solid medium (PDA liquid medium containing 2.0 % agar), modified S-7 medium based on S-7 medium with the addition of tyrosine, linoltic acid and phenylalanine at the final concentrations of 1.5, 1.5 and 5.0 mg/l, respectively (Zhao et al. 2008b), LB liquid medium (Shen et al. 2000), and LB solid medium (LB liquid medium with 1.5 % agar) were sterilized under 1.05 kg/cm^2 pressure for 20 min, cooled to 50-55 °C, then ampicillin was added at the final concentration at 100 μg/ml (Zhao et al. 2008b). SOC medium (100 ml) was prepared as follows: 2.0 g tryptone, 0.5 g yeast extract, 1.0 ml 1.0 mol/l NaCl, 0.25 ml 1.0 mol/l KCl, adjusted to pH 7.0, sterilized under 1.05 kg/cm^2 pressure for 20 min, then 1.0 ml 2.0 mol/l Mg^{2+} accumulator and 1.0 ml 2.0 mol/l glucose were added after filter-sterilization.

Primers

Four anchor primers in mRNA differential display, 5′-oligo dT11 MA - 3′, 5′-oligo dT11 MT - 3′, 5′-oligo dT11 MG - 3′, 5′-oligo dT11 MC - 3′ and 20 random primers were utilized in mRNA differential display. The primers were synthesized by TaKaRa Company (Dalian, China).

Culture of HDF-68 and extraction of products

The strain HDF-68 was activated on PDA slope culture at 28 °C, then transferred into 50 ml PDA liquid medium in 250 ml flask and cultured at 28 °C for 3 day. The cells were inoculated into modified S-7 culture at the concentration of 3 % (v/v) and fermented at 28 °C and 150 rpm for 12 day. At the end of fermentation, the filtrate and mycelium were collected. The filtrate was extracted twice using acetic ether, 1 hour each time, and supernatants were collected. The mycelium was whetted fully and extracted using 30 ml acetic ether for 1 h. The organic phase was combined and then distilled to remove the organic solvent (Zhao et al. 2008b). Three replicate cultures were used for the analysis.

Analysis of differential gene expression of strain HDF-68 in different taxol-synthesis phases

Culture and collection of mycelium

Culture and collection of mycelium were carried out as previously described (Zhao et al. 2008b).

Extraction of total RNA and removal of residual DNA

Based on our previously work, we have determined that culture day 2 is regarded as a representative non-taxol-synthesis phase point (Chi et al. 2008). Hence, we selected total RNA from the fermentation broth of the strain HDF-68 at day 2, day 6, day 8 and day 10 according to the manufactory instruction of Reagents (Promega, USA). Total RNA was ablated of residual DNA by RNase-free DNase I, extracted by phenol and chloroform, and recovered by ethanol. RNA integrity was checked by 1.0 % denatured agarose gel electrophoresis, and RNA quality and concentration were analyzed by a spectrophotometer (A_{260}/A_{280}, A_{260}/A_{230} ratio).

mRNA differential display

Using oligo dT11 MA, oligo dT11 MT, oligo dT11 MG and oligo dT11 MC as the anchor primers and total RNA in non-taxol–synthesis phase and taxol-biosynthesis phase as templates, first strand cDNA was synthesized with M-MLV reverse transcriptase. The reaction product was diluted ten times with sterile double distilled water, and then amplified in a DNA Thermal Cycler using primers that were combined with corresponding anchor primers and one of the 20 random primers (P1-P20), as shown in Table 1.

PCR reactions were carried out as five cycles of heat treatment at 94 °C for 1 min, 94 °C for 30 s, 40 °C for 1 min, and 72 °C for 1 min, a total of 35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 1 min, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The PCR products were stored at 4 °C. After PCR amplification, the amount of target cDNA in the control and treated samples was compared by fluorescence monitoring of each cycle (Italia et al. 2011). Amplification products were resolved on a 6.0 % denaturing polyacrylamide gel with an electrophoresis buffer of 1×TBE, 300 V for 30 min, and then 500 V until the xylene cyanol was close to the bottom. Bands were displayed with silver staining.

Table 1 Sequences of random primers

Code	Sequence	Code	Sequence
P1	5′- AAACTCCGTC −3′	P11	5′- GATCAATCGC −3′
P2	5′- TGGTAAAGGG −3′	P12	5′- TCGGTCATAG −3′
P3	5′- GATCATGGTC −3′	P13	5′- GATCTGACTG −3′
P4	5′- TTTTGGCTCC −3′	P14	5′- TCGATACAGG −3′
P5	5′- GTTTTCGCAG −3′	P15	5′- TACAACGAGG −3′
P6	5′- TACCTAAGCG −3′	P16	5′- GATCAAGTCC −3′
P7	5′- GATCTGACAC −3	P17	5′- GATCTCAGAC −3′
P8	5′- GATCTAACCG −3′	P18	5′- GGTACTAAGG −3′
P9	5′- TGGATTGGTC −3′	P19	5′- GATCACGTAC −3′
P10	5′- GGAACCAATC −3′	P20	5′- CTTTCTACCC −3′

Recovery, reamplification and cloning of differential bands

Differential bands were excised from denaturing polyacrylamide gel for recovery of DNA using the method of Hao et al. (2002). Excised bands were amplified with the same primers and reaction conditions as described above. Amplification products were cloned into pGEM-T Vector and transformed into *E. coli* $DH_{5\alpha}$. The clones were screened by blue and white patches, then identified according to BstZI digestion and PCR detection

Reverse dot blot hybridization of differential fragments

After digestion, the recombinant plasmids were resolved on a 1.5 % agarose gel. Inserted fragments were recovered and labeled with DIG by DIG high prime DNA labeling and detection starter kit II. Respective 20 μg total RNA in non-taxol–synthesis phase and taxol-biosynthesis phase were transferred to Hybond-N^+ nylon membrane after resolving on a 1.5 % agarose gel and detected according to instructions of DIG high prime DNA labeling and detection starter kit II for hybridization and chromogenic detection. The above-mentioned cloned fragments were used as a probe.

Sequencing and homology analysis

Sequencing of the PCR products was performed by Shanghai Biological Engineering Company Ltd. Fragments was sequenced using an ABI PRISM 377–96 DNA Sequencer according to the manufacturer's instructions. The sequences obtained were analyzed by homology comparison to all non-repetitive sequences in GenBank, European Molecular Biology Laboratory (EMBL), DNA Data Bank Of Japan (DDBJ) and Protein Data Bank (PDB) database using Blast (http://www.ncbi.nlm.nih.gov). ORFs of new sequences were analyzed using NCBI/ORF finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Derived amino acid sequences were analyzed by homology comparison to all of the proteins in GenBank, CDS translation, PDB and Protein Information Resource (PIR) using BLAST tools.

Results

Analysis of differential gene expression of HDF-68 in different taxol-synthesis phases

As shown in Fig. 1, each lane displayed 30–40 clear bands, and the amplified cDNA bands of primer T11MA reverse transcription product were more than others. Moreover, different arbitrary primers amplified a different number of cDNA bands. The lengths of the amplified cDNA fragments ranged

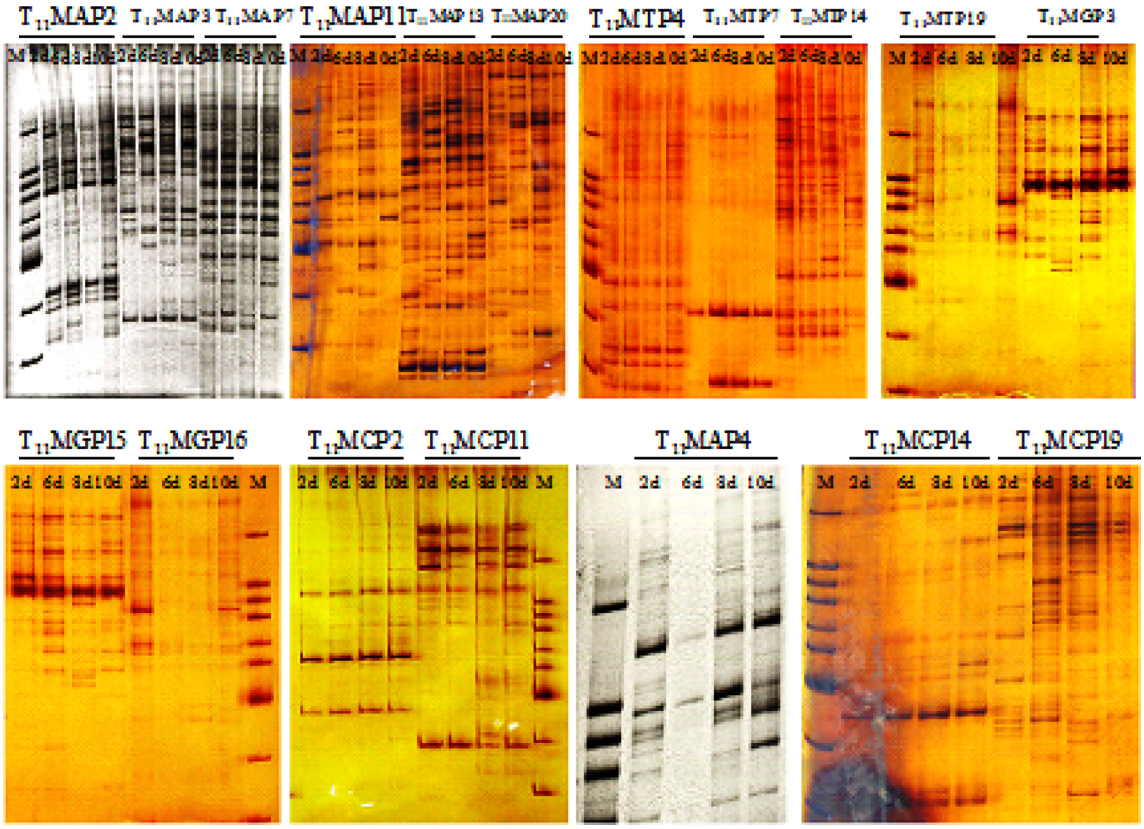

Fig. 1 Differential display patterns of mRNA from strain HDF-68 in taxol-biosynthesis phase and non-taxol-synthesis phase. M represents 100 bp DNA Marker; 2 day represents the non-taxol-synthesis phase; 6 day/8 day/10 day represent the taxol-biosynthesis phase

from 0.2 kb to 1.5 kb, with most of them between 0.4 kb and 1.0 kb.

mRNA expression of strain HDF-68, which maintained mycelia basic physiological activities in different taxol-synthesis phases, was similar. About 20 % of the cDNA band had qualitative and quantitative differences. Qualitative differences could be found in new bands and the disappearance bands. Differences in the quantity at different times could be found in enhanced expression or decreased expression. Some of the differentially expressed genes might be related to the aging of mycelia, and other genes might be involved in the synthesis of taxol, i.e., candidate genes for the endophytic fungi taxol biosynthesis genes.

Differential genes expression from HDF-68 in different taxol-synthesis phases

As shown in Fig. 2, differential gene expression from strain HDF-68 in different taxol-synthesis phases was divided into: synthesis phase differentially expressed genes (A), non-synthesis phase differentially expressed genes (B); regulated expression genes (C); and down-regulated expression genes (D) from non-synthesis phase to synthesis phase.

Recovery and reamplification of differential bands

In order to screen taxol-synthesis–related genes from the differentially expressed genes, specific expression fragments or significantly enhanced expression fragments in synthesis phase were recovered in the study. As shown in Fig. 3, 62 differential bands were recovered, and 38 differential bands that were marked R1-R38 obtained amplification products during second PCR amplification analysis. The differential expression characteristics of recovered fragments are shown in Table 2.

Reverse dot blot hybridization of differential fragments

RNA samples extracted from broth of the strain HDF-68 at day 2, day 3, day 4, day 6, day 8, day 10, day 12 and day 14 were used as templates for producing cDNA probes. As shown in Fig. 4, Northern analysis showed that out of the amplified 38 fragments, 22 fragments were false-positives and the false-positive rates were 57.9 %; for 16 fragments, hybridization signal and its strength were the same as the results of DDRT-PCR, and these fragments were marked as TBR1–TBR16. Cloning, sequencing and homology analyses of TBR1–TBR16 were conducted.

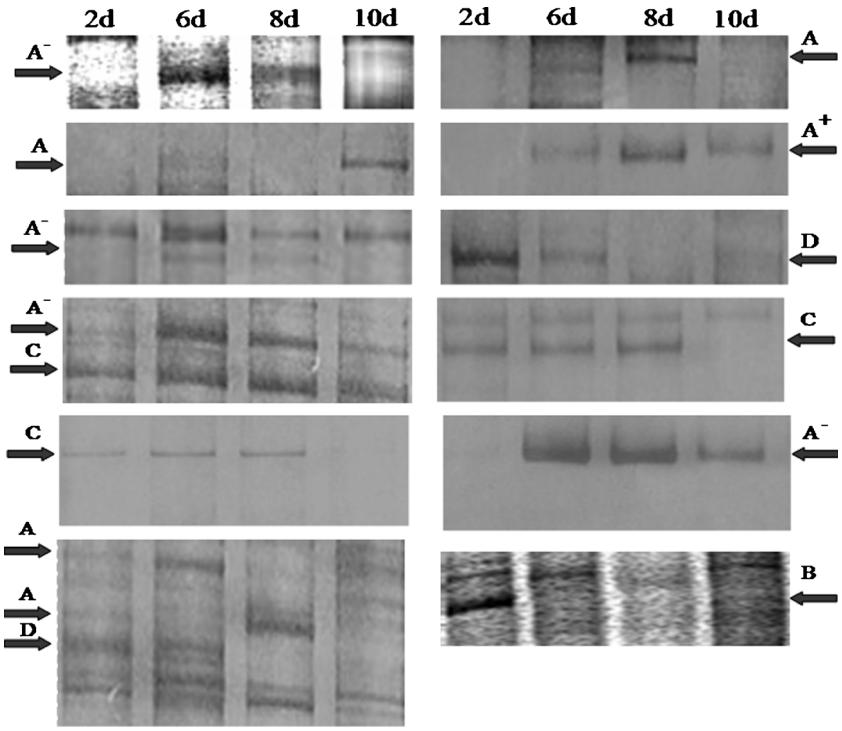

Fig. 2 Genes of differential expression from strain HDF-68 in taxol-synthesis phase and non-taxol-synthesis phase. A represents genes of specific expression in taxol-synthesis phase; A^+ represents genes of enhanced expression in taxol-synthesis phase; A^- represents genes of decreased expression in taxol-synthesis phase; B represents genes of specific expression in non-taxol–synthesis phase; C represents genes of enhanced expression from non-taxol–synthesis phase to taxol-synthesis phase; D represents genes of decreased expression from non-taxol-synthesis phase to taxol-synthesis phase

Cloning and homology analysis of cDNA fragments

The sequencing results suggested that the 5′ and 3′ of each fragment were the same as the chosen random primer sequences. Homology analysis of 16 EST fragments with NCBI Blast program showed that nucleic acid sequences of TBR3, TBR4 and TBR7 were extremely similar to the corresponding human sequences (Table 3). It was speculated that these ESTs were conservative and associated with the life cycle. The genes of cytochrome oxidase subunit I in TBR11 and *Neurospora crassa* mitochondrial were quite similar (Score = 186 bits, Identities = 63 % (75/119), Expect = 4e − 45). As known, several cytochrome oxidases participated in the cell-mediated biosynthesis of taxol from yew, but no similar cytochrome oxidases genes were isolated from yew trees. There was information available about other ESTs because the functions of their homologous sequences were unknown. Half of the ESTs did not show homology to existing sequences, indicating that they represent new genes. At present, the results of the test show that there are few molecular biology studies on the *N. sylviforme* taxol-producing fungus and similar species.

Through analyzing the EST fragments, one or more ORFs were found in all of the 16 ESTs (Table 4). This suggests that obtained EST fragments were in the coding region of the gene. Meanwhile, our study also confirmed that there was more information about amplification product in 3′-UTR region when single random primers were used instead of the anchor

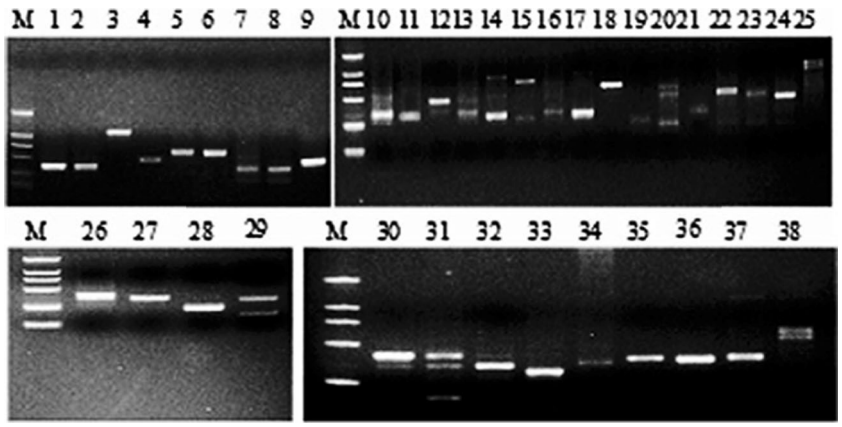

Fig. 3 Electrophoresis of reamplified products of the differential fragments. M represents DNA Marker DL2000

Table 2 Differential expression characteristics of recovered fragments

Code	2 day	6 day	8 day	10 day	Code	2 day	6 day	8 day	10 day
R1		+	+	+	R20		+	++	
R2			++	++	R21	+	++	++	
R3			+	++	R22		+	+	+
R4		+			R23			+	
R5		+++	++	+	R24		++	+	
R6			+	++	R25				+
R7	+	+++	++	++	R26		++	++	++
R8		++	+++		R27			++	++
R9		+	+	+	R28				+
R10	+	+	++	++	R29	+	+	++	++
R11		+	+++		R30		+	++	+
R12		+	+	+	R31			+	++
R13		++		+	R32		++	+	
R14		++	++	++	R33		+	+	
R15		+			R34		+	+	+
R16		+	++	+	R35			+	+
R17			+	+	R36		++	++	++
R18	+	+	++		R37	+	+	++	+
R19		+	++	+++	R38		+	++	

"+" indicates presence of a band and number of "+" indicates band brightness

primers or random primer combinations in DDTR-PCR. Homologous proteins of seven EST encoded products were found in the protein database. However, only homologous proteins of TBR10 and TBR11 are known; others belong to hypothetical proteins.

Discussion

Traditional mutation breeding method is normally used for strain selection, but there are some disadvantages of this method, such as low mutagenic rate, superabundant screening, long cycle and some accumulation of irrelevant or negative mutations caused by repeated mutagenesis procedure. For this reason, the strain is insensitive to mutagenesis conditions, and it is difficult to increase products using the traditional mutation breeding method. Strain degeneration even occurs in growth rate, assimilation of substrates and environmental tolerance. The screening method of industrial strains is changing from traditional mutation breeding to genetic engineering, and from metabolic engineering to transformation of strains (Stephanopoulos and Alper 2004; Stephanopoulos et al. 2004; Liu and Tang 2005). However, the successful implementation of the latter is dependent on a detailed

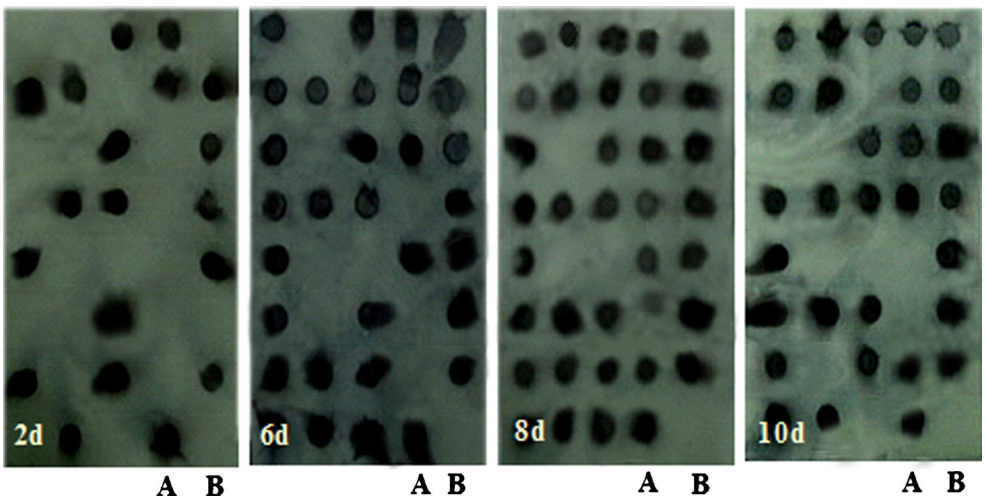

Fig. 4 Reverse Northern blot hybridization analysis of recovered differentially expressed cDNA fragments. *A* represents positive control; *B* represents negative control

Table 3 Homology analysis of obtained EST fragments with NCBI Blast program

EST	Homologous sequences[a]	Score value	E value	Identities
TBR1	Not found			
TBR2	Not found			
TBR3	Human DNA sequence from clone RP11-812I20 on chromosome 6, complete sequence	890	0.0	469/478 (98 %)
TBR4	Homo sapiens differential display (Yong LL) Homo sapiens cDNA clone dominant regulation-6	111	9e-22	56/56 (100 %)
TBR5	Not found			
TBR6	Not found			
TBR7	Homo sapiens chromosome 19 clone CTB-184G21, complete sequence	878	0.0	459/463 (99 %)
TBR8	Not found			
TBR9	Not found			
TBR10	*Mycobacterium tuberculosis* H37Rv complete genome; segment 9/13	198	6e-91	73/85 (85 %)
TBR11	*Neurospora crassa* mitochondrial COI gene for cytochrome oxidase subunit I (exon 1–5)	186	4e-45	
TBR12	Not found			
TBR13	*Staphylococcus aureus* subsp. *aureus* Mu50 genomic DNA, Section 8/9	61.9	6e-07	61/71 (85 %)
TBR14	Not found			
TBR15	Hypocrea jecorina cDNA clone tric036xk19	65.9	5e-08	57/65 (87 %)
TBR16	Gibberella zeae PH-1 chromosome 3 strain PH-1; NRRL 31084	73.8	3e-10	67/77 (87 %)

[a] Only the most highly homologous sequences are listed in the Table 3

understanding of the molecular basis of product biosynthesis and genetic regulatory mechanisms.

Primary strain HQD_{33} belonging to *N. sylviforme* is a newly recorded *genus*. There is little information on *N. sylviforme,* and test strains HDF-68 are high-yield fusants of protoplast fusion with strains $UV_{40\text{-}19}$ and $UL_{50\text{-}6}$; it is too difficult to use a traditional mutation breeding method. Studies of taxol biosynthesis genes are important to clarify the taxol biosynthetic pathways and construct high-yield genetically engineered strains. Although there has been great progress in the cloning and identification of related enzyme genes during the cell-mediated biosynthesis of taxol from yew, to date, there are no reports about isolating taxol biosynthesis-related genes. In summary, metabolic regulation for successfully increasing taxol yield is dependent on isolation and functional analysis of fungal taxol biosynthesis genes.

There are many methods of cloning genes, such as reverse cloning and homologous PCR. However, separation of differentially expressed genes has a great advantage, because there is little information on the genetic background of taxol-producing fungi. Since Liang and Pardee established the mRNA differential display (DDRT-PCR) (Liang and Pardee 1992) method, it has been used by many laboratories in the area of biomedical research (Hu et al. 2004). It is one of the major tools for discovering genes whose expression levels are altered under different conditions (De Luca et al. 2011). Disadvantages include a high false positive rate of up to 50-75 % (Debouck 1995; Wan et al. 1996), relatively small, amplified differential bands (usually between 100 and 500 bp), and most of the obtained cDNA fragments are in the 3′ end of non-coding sequence. The following measures were taken to overcome the above-mentioned problems. Firstly, genomic DNA was removed from RNA samples during DNase treatment of total RNA, because trace amounts of genome DNA in the RNA samples could combine with the random primer and increase the number of short non-specific bands and false positive clones. Secondly, a two-stage PCR amplification procedure was carried out, amplifying at 40 °C for five cycles, then proliferating for 35 cycles after the annealing temperature was increased to 45 °C. This method is for amplification efficiency, and also amplification specificity and reproducibility. This study also showed that the amplification efficiency is different between vermiform anchor primers and random primers, because of the different characteristics of the template sequences. Furthermore, different PCR procedures had different effects due to different combinations of primers, so the best amplification procedure needs to be determined by optimization. Third, this test combined a random primer instead of the anchor primer with other random primers in the PCR amplification reaction. However, preliminary experiments showed that two random primers led to fragments on the gel due to the short bands.

The results of this study showed that rich and clear bands under low background signal could be obtained after polyacrylamide gel electrophoresis and silver staining. In this method, cDNA was divided into four categories after

Table 4 Homology comparison of cloned cDNA fragments with nucleic acid sequences in GenBank

Sequence name	Phase	The beginning and end site of coding region	The length of coding region	The number of amino acids	Homology analysis
TBR1	+2	56–181	126	41	No homologous protein found
	−1	168–278	111	36	No homologous protein found
	−3	220–324	105	34	No homologous protein found
	−2	29–130	102	33	No homologous protein found
TBR2	−2	2–205	204	67	No homologous protein found
	+1	217–351	135	44	No homologous protein found
	−3	247–351	105	34	No homologous protein found
TBR3	−3	1–149	149	49	No homologous protein found
	+1	322–447	126	41	Retrovirus-related hypothetical protein II 71 % (29/41)
	+2	2–127	126	41	No homologous protein found
TBR4	−2	84–293	210	69	No homologous protein found
	+1	142–344	204	67	No homologous protein found
TBR5	−2	31–300	270	89	No homologous protein found
	+2	122–292	171	56	No homologous protein found
	+1	148–300	153	50	No homologous protein found
TBR6	−1	1–111	111	36	No homologous protein found
TBR7	−3	201–461	261	86	hypothetical protein-human unnamed protein product 69 % (49/71)
	+2	209–361	153	50	No homologous protein found
	+3	75–212	138	45	No homologous protein found
	−2	109–222	114	37	No homologous protein found
TBR8	+2	326–450	126	41	No homologous protein found
	+3	3–119	117	38	No homologous protein found
	−2	310–414	105	34	No homologous protein found
TBR9	+3	162–305	144	47	No homologous protein found
TBR10	+1	25–479	456	151	RelA (*Mycobacterium avium* subsp. *paratuberculosis str.* K10) 85 % (133/156)
	−3	140–295	156	51	No homologous protein found
	−1	133–261	129	42	No homologous protein found
TBR11	+1	64–279	216	71	Gene coI intron 1 protein (*Neurospora crassa* mitochondrial) 64 % (46/71)
	+3	3–116	114	37	No homologous protein found
TBR12	−3	153–254	102	33	No homologous protein found
TBR13	+3	96–281	187	62	Conserved hypothetical protein (*Staphylococcus aureus* subsp. *aureus* Mu50) 70 % (36/51)
	−3	35–145	111	36	No homologous protein found
	+1	1–111	111	36	Hypothetical protein (*Staphylococcus aureus* subsp. *aureus* MW2) 83 % (30/36)
TBR14	−3	1–248	248	82	No homologous protein found
	−1	245–369	126	41	No homologous protein found
TBR15	−3	1–288	288	95	Hypothetical protein (related to BCS1 protein precursor, *Neurospora crassa*) 86 % (83/96)
	−1	75–221	147	48	No homologous protein found
	−1	255–359	105	34	No homologous protein found
TBR16	−1	3–533	531	176	Hypothetical protein (*Neurospora crassa*) 82 % (145/176)
	+2	296–541	246	81	No homologous protein found
	−2	368–475	108	35	No homologous protein found
	+3	210–214	105	34	No homologous protein found

transcription by anchor primers, then PCR amplification procedures were started by using a random primer. The length of most amplification products fell between 0.4 kb and 1.0 kb, and some of them were more than 1.5 kb, which was significantly longer than fragments obtained by the conventional mRNA differential display approach. Moreover, the limitations of information about products in the 3′ non-coding region were addressed and the available information was increased using the random primer amplified fragments. In short, the single arbitrary primers mRNA differential display (SAP-mRNA DD) could be used for studying gene differential expression and to overcome some of the shortcomings associated with conventional mRNA differential display.

The study has also compared gene differential expression in every stage of the taxol synthesis process in the endophytic fungus HDF-68. Comparison of samples could reduce the false positive rates so that it was useful for screening genes related to taxol synthesis, and directly related synthesis genes should show sustained expression in the taxol synthesis period. The results indicated that the types and levels of the differentially expressed genes were different, as well as the complexity of the taxol biosynthesis process. Specifically expressed genes and enhanced expression genes in the taxol synthesis phase might be closely related to the synthesis of taxol.

The main problem of mRNA DDRT-PCR is the high false positive rate (Ahmed et al. 2011), so identification of differential fragments is crucial. Northern hybridization is a classic and reliable way to identify differences in mRNA levels, but every differential fragments. In addition, low abundance mRNA molecules are difficult to detect. Reverse Northern techniques using labeled cDNA as a probe to hybridize with differential fragments greatly improved the efficiency of hybridization. This study also applied the reverse Northern hybridization to identify all recovered fragments, using four labeled cDNA fragments. Hybridization results showed 57.9 % false positive rates, which confirmed the high false positive rates associated with mRNA differential display.

The study obtained 16 positive clones through analyzing differential gene expression patterns in different stages of *N. sylviforme* taxol synthesis. However, these ESTs acquired only represented parts of the corresponding cDNA sequences. Therefore, it will be necessary obtain full-length genes of these ESTs by rapid amplification of cDNA ends (RACE) in future experiments, and it will be informative to confirm the function of these genes. There are still other issues that need to be addressed, such as whether these genes are the key enzymes in the taxol biosynthetic pathway of endophytic fungi compared with cell culture producing taxol. This study provides valuable clues for identification of functionally related genes in taxol-producing endophytic fungi and for constructing bioengineering strain with high taxol yield.

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