

IDENTIFICATION OF WOOD DECAY RELATED GENES FROM *PIPTOPORUS BETULINUS* (BULL. FR.) KARSTEN USING DIFFERENTIAL DISPLAY REVERSE TRANSCRIPTION PCR (DDRT-PCR)

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ABSTRACT

Piptoporus betulinus (Bull. Fr.) Karsten causes brown rot on the wood of weakened birch species. Little work has been published on the molecular mechanism of its wood decaying. In this study differential display reverse transcription PCR (DDRT-PCR) was used to isolate wood decay genes from *P. betulinus*, which were induced in the wood samples of white birch. We found that 29 differentially expressed cDNA bands (~4.7%) were especially specific to cDNA samples from the mycelia of *P. betulinus* inoculated wood samples. Additionally, 12 transcripts from *P. betulinus* were induced during wood decay. These 12 clones were isolated, cloned and sequenced. Seven of them were highly homologous to known genes including pre-mRNA splicing factor *syf2*, arabinose 5-phosphate isomerase, alpha/beta hydrolase, lytic transglycosylase, polysaccharide deacetylase, inositol monophosphatase and glucoamylase-like protein. These results suggested that the process of decaying birch wood by *P. betulinus* was intricate, involving enzyme or protein changes.

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Introduction

Wood is an important renewable and natural resource with a number of uses (13). Wood decay incurs great losses annually (29). Many fungi can usually decay wood and identification of related genes from such fungi can help to better understand the roles different fungi play in the decay process. Among wood-rotting fungi, *Piptoporus betulinus* (Bull. Fr.) Karsten as a brown-rot fungus is a main factor in decaying birch (*Betula platyphylla* Suk.) wood. This fungus decays wood slowly under many conditions and causes brown rot on the branch and trunk wood of weakened birch trees (6). To degrade wood, this fungus first decays the inner discolored trunk zone, and then the non-discolored wood (28, 36). This decay is a complex process involving many factors. However, the exact molecular mechanism of *P. betulinus* Karsten decay still remains hypothetical and controversial. To date, many lignin decay genes from fungi have not been detected (27).

During the process of wood decay, a series of physiological and biochemical changes take place that lead to the induction of specific fungal genes (32, 34). To determine which genes are differentially expressed, comprehensive analysis is essential. This can be achieved by differential display reverse transcription PCR (DDRT-PCR) (1, 2, 7). This technique has been widely used, as it is a simple method which does not require previous genomic information about the species (8, 10, 12, 14). The transcriptome of a given organism can change in response

to different conditions. Because strong gene expression results in abundant mRNA and includes all transcripts in the cell, transcriptomics is complex (22). The transcriptome reflects the genes that are being actively expressed at some time and can provide some useful information about the fungal activity in decaying wood (21).

In this study, changes in gene expression of the mycelium of *P. betulinus* inoculated wood and mycelium of pure culture were analyzed by DDRT-PCR to identify genes related with birch wood decay. Accordingly, these genes were demonstrated by using reverse northern blot analysis. This approach led to the identification of novel *P. betulinus* genes expressed during birch wood decay.

Materials and Methods

Fungal isolation and culture conditions

The wood wafer samples were from birch (*Betula platyphylla* Suk.) tree collected from Maoershan, China in September 2005. This wood was cut into wafers (3 × 1 × 2 mm) and stored at -20 °C until use. *P. betulinus* Karsten was isolated from young fresh fruit bodies growing on a birch tree in Maoershan, China in October 2005. The identification of fungal species was done on the basis of macro- and micro-morphological features of the fruit bodies, as well as size and color of the colony, type and diameter of hyphae (5, 15). To obtain abundant mycelia, the fungi were cultured in King B medium (protease peptone (Shanghai Biotech Co. Ltd., Shanghai, China) 2%, glycerol 1%, K₂HPO₄ 0.15%, MgSO₄ 0.15%, agar 1.5%, distilled H₂O, pH 6.9 and Difco nutrient agar, pH 6.8), and incubated at 28 °C for 30 days.

After birch wood samples were autoclaved at 120 °C for 20 min, they were transferred onto petri dishes (90 mm in diameter) filled with abundant mycelia. The plates were incubated in darkness for 60 days at 25 °C. The mycelia from *P. betulinus* inoculated wood and the mycelia from pure culture were used as treatment and control, respectively.

Extraction of total RNA and differential display reverse transcription RT-PCR (DDRT-PCR)

Extraction of total RNA was performed according to the method described by Qian et al. (30). For the first strand cDNA synthesis, the 20 µl reaction mixture contained 8 µg of DNA-free total RNA, 4 µl of 10× AMV buffer, 2 µl of 10 mM dNTP, 2 µl of 20 µM anchor primer (M1, M2 and M3, **Table 1**), 40 U of RNasin Ribonuclease inhibitor and 1 µl of AMV reverse transcriptase (200 U/µl) (Promega GmbH, Mannheim, Germany). Before the reverse transcriptase was added, the mixture was incubated at 70 °C for 10 min, and cooled in ice for 15 min. Then the mixture was incubated at 42 °C for 75 min, 45 °C for 10 min, 70 °C for 10 min. The reaction was stopped by incubation at 95 °C for 5min and the reaction tubes were stored at -20 °C until use.

PCR amplification was performed in a final volume of 20 µl containing 12 µg of cDNA from the reverse transcription reaction, 2 µl of 2 µM dNTPs, 2.0 µl of 10× *Taq* buffer, 2.0 µl of 25 mM MgCl₂, 2.0 µl of 20 mM arbitrary primer (S1-S26, **Table 1**), and 1.25 U of *Taq* DNA polymerase (TaKaRa Biotechnology, Dalian, China). The following PCR program was used: 94 °C for 3 min, 40 cycles at 94 °C for 30 s, 36 °C for 1 min, 72 °C for 50 s and a final extension step at 72 °C for 7 min.

The 20 µl PCR reaction mixtures were mixed with 5 µl of gel loading dye and then preheated at 95 °C for 5 min. The DDRT-PCR products were separated on a 6% polyacrylamide gels for 6 h at 400 V and visualized by silver staining (19).

Both reverse transcription and PCR reactions were carried out five times to detect false-positive staining.

The differentially expressed bands were excised using a sharp, clean razor blade, and purified using the Poly-Gel DNA Extraction Kit (Omega, USA), then cloned in pGEM-T Easy vector (Promega GmbH, Mannheim, Germany). Sequencing was performed at Genes Build the Future (BGI, Beijing, China). Database searches and comparison with published sequences were carried out using the GenBank/EMBL databases.

Reverse northern blot analysis

The probes were labeled with the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) following the manufacturer's instructions. The clones showing differential expression were blotted on nylonHybond-H⁺ membranes (GE, USA) at 50 °C for 30 min. Then the membranes were subjected to hybridization with total DIG-labeled cDNA probes of mycelia RNA from *P. betulinus* inoculated wood and mycelia from pure culture, respectively.

Results and Discussion

After screening 78 primer combinations (3 anchored primers and 26 arbitrary primers), a total of 31 primer combinations were used to amplify cDNA samples obtained by reverse transcription of total RNA. A representative gel from a differential display experiment by M3-S22 primer combinations was shown in **Fig. 1**. A total of 620 resolvable bands were generated with an average of 20 bands per primer combinations. Twenty-nine differentially expressed cDNA bands (~4.7%) were especially specific to cDNA samples from the mycelia of *P. betulinus* inoculated wood. These bands were 100 to 800 bp in length.

TABLE 1

Sequences of the anchor primers and arbitrary primers used (2)

Arbitrary primer	Sequence (5'-3')	Arbitrary primer	Sequence (5'-3')	Arbitrary primer	Sequence (5'-3')
S1	TACAACGAGG	S11	TACCTAAGCG	S21	GATCTAACCG
S2	TGGATTGGTC	S12	CTGCTTGATG	S22	GATCGCATTG
S3	CTTCTACCC	S13	GTTTTTCGCAG	S23	GATCTGACTG
S4	TTTTGGCTCC	S14	GATCAAGTCC	S24	GATCATGGTC
S5	GGAACCAATC	S15	GATCCAGTAC	S25	GATCATAGCG
S6	AAACTCCGTC	S16	GATCACGTAC	S26	GATCTAAGGC
S7	TCGATACAGG	S17	TCGGTCATAG	Anchor primer	Sequence (5'-3')
S8	TGGTAAAGGG	S18	GATCTCAGAC	M1	AAGCTTTTTTTTTTC
S9	GATCTGACAC	S19	GATCATAGCC	M2	AAGCTTTTTTTTTTTA
S10	GGTACATTGG	S20	GATCAATCGC	M3	AAGCTTTTTTTTTTTG

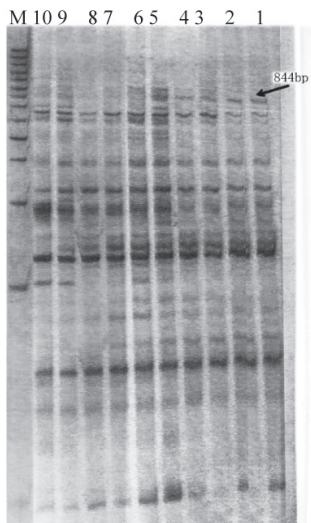


Fig. 1. Pattern of mRNA differential display visualized by silver staining. RT-PCR banding patterns obtained with the M3-S22 primer combinations. Arrows indicate differentially expressed cDNA fragments that were recovered from gel and analyzed further. Lanes 1-5: treatment samples; lanes 6-10: controls; lane M: 100 bp DNA Ladder Marker.

To bias against isolating differential display false-positive cDNA, 29 different bands were used for reverse northern blot analysis using as probes cDNAs from total RNA isolated from the mycelia of *P. betulinus* inoculated wood and mycelia of pure culture, respectively (**Fig. 2**). In this study, 10 blots hybridized neither to treatment cDNA nor to control. Seven blots hybridized to both cDNA. The 10 non-hybridized blots were defined as false positives. Twelve blots (1A1, 1A6, 1B2, 1B5, 1B6, 1B7, 1C2, 1C3, 1C5, 1D1, 1D2 and 1D4) hybridized to treatment cDNA specifically. These specifically expressed cDNA bands were detected, successfully extracted from the gels, and cloned. There is limited data on regulated genes isolated from *P. betulinus* mycelia. Our previous studies showed that the action of *P. betulinus* during the process of birch wood decay is intricate, involving changes of the ligninolytic enzymes including laccase and Mn peroxidase (31). Several investigations suggested that new fungal mRNA transcripts were suppressed or induced during wood decay (17,

19, 35). However, the regulation mechanism of *P. betulinus* (Bull. Fr.) Karsten wood decay is still unclear.

In the present work, to gain a better understanding of the molecular mechanisms underlying birch wood decay, we cloned 12 cDNA fragments from *P. betulinus* from decaying birch wood by DDRT-PCR. Twelve specifically expressed cDNA bands were sequenced and high quality sequences were obtained. The obtained sequences were compared against the NCBI/EMBL database. Only 5 clones (1A6, 1B2, 1B6, 1C5 and 1D4) were found not to have any homologous sequences in the database, the other 7 clones (1A1, 1B5, 1B7, 1C2, 1C3, 1D1 and 1D2) were highly homologous to known genes including pre-mRNA splicing factor syf2, arabinose 5-phosphate isomerase, alpha/beta hydrolase, lytic *trans*-glycosylase, polysaccharide deacetylase, inositol monophosphatase and glucoamylase-like protein. The results of these analyses are presented in **Table 2**.

Because reverse northern blot analysis appears to be a more sensitive assay than conventional northern blotting (27), these cDNA fragments were further confirmed using reverse northern blot to detect false positives. In this study, the RNA blot analysis revealed that 12 clones were specifically induced in wood decay by *P. betulinus*. As demonstrated in **Table 2**, five isolated cDNAs shared no homology to any known sequences in the GenBank/EMBL databases (1A6, 1B2, 1B6, 1C5 and 1D4). Six genes were found to be similar to molecules of the following functions: gum, cellulose and hemicellulose degradation (1B5, 1C2, 1C3 and 1D1), and carbohydrate metabolism (1B7 and 1D2).

According to the sequence similarity analysis of the 1A1 clone it was suggested that it encodes a putative pre-mRNA splicing factor syf2. The pre-mRNA splicing factors are ubiquitous; the spliceosome contains five small nuclear RNAs and more than fifty proteins (35). Over the past years, considerable progress has been made toward understanding how these factors function to achieve fidelity in splicing (9). These pre-mRNA splicing factors are modulators of RNA structures with potential roles in gene expression, transcription, nuclear and mitochondrial RNA splicing, ribosome synthesis, translation, RNA editing, mRNA export, and mRNA turnover

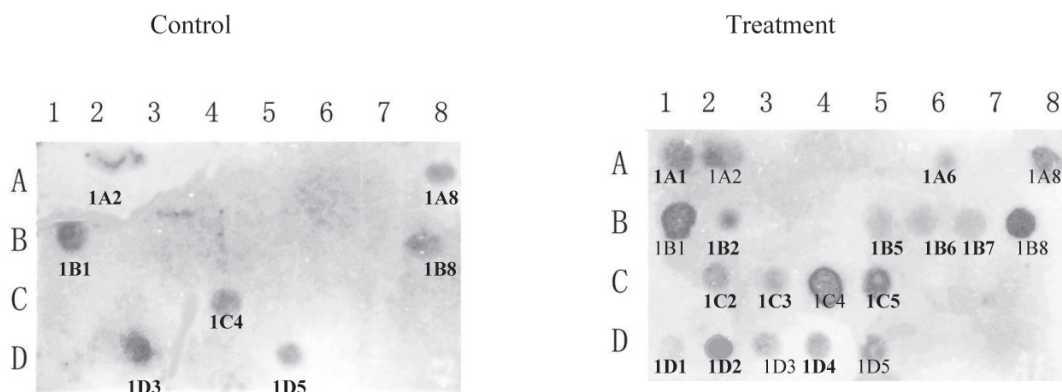


Fig. 2. cDNA Northern blot patterns of 29 clones. Total RNA was isolated from the mycelia of *P. betulinus* inoculated wood (treatment) and mycelia of pure culture (control), respectively.

DDRT-PCR transcripts identified by sequence homologies found in the NCBI/EMBL sequence database

Clone number	GenBank accession number	Primer combination	Size (bp)	Blast homology	Identity (%)	General function
1A1	NW_001849826	M1-S1	754	pre-mRNA splicing factor syf2	70	pre-mRNA splicing
1A6		M1-S18	493	No sequence was obtained	0	unknown
1B2		M2-S5	152	No sequence was obtained	0	unknown
1B5	CP000031	M2-S7	271	arabinose 5-phosphate isomerase	95	gum and hemicellulose degradation
1B6		M2-S17	285	No sequence was obtained	0	unknown
1B7	NM018811	M2-S17	405	alpha/beta hydrolase	66	fat metabolism
1C2	CP000620	M2-S24	233	lytic transglycosylase	72	cell wall degradation
1C3	NZ_ABTA01000002	M3-S14	179	polysaccharide deacetylase	51	cellulose degradation
1C5		M3-S19	422	No sequence was obtained	0	unknown
1D1	CP000390	M3-S22	792	inositol monophosphatase	62	cellulose degradation
1D2	CP000240	M3-S24	352	glucoamylase-like protein	58	starch metabolism
1D4		M3-S26	648	No sequence was obtained	0	unknown

(3, 4). However, it is not known how this gene may participate in birch wood decay. Therefore, further studies are necessary to understand its possible functions related to degradation during wood decay.

Clone 1B5, encoding a putative arabinose 5-phosphate isomerase, played a purely catabolic role in ribose 5-phosphate metabolism by converting ribose 5-phosphate to ribulose 5-phosphate (20). To date 770 cDNA clones of arabinose 5-phosphate isomerase have been isolated. It is thought that the products of these genes may take part in the pentose-phosphate pathway involved in arabinose metabolism (5). However, the function of this isomerase is far from clear in wood decay by *P. betulinus*.

The isolated gene 1B7 codes the alpha/beta hydrolase fold, also called phosphatase B, which is common to a number of hydrolytic enzymes of different phylogenetic origin and catalytic function (18). EST database showed that the core of the alpha/beta hydrolase fold is an alpha/beta sheet (24). However, the function of these hydrolase is far from clear in wood-decay fungi.

Clone 1C2 codes for lytic *trans*-glycosylases participating in cytolysis (26). Previous studies demonstrated that lytic *trans*-glycosylases catalyse the cleavage of the β -1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine and belong to the family of murein hydrolases that are involved in the maintenance of cell-wall integrity during cell elongation and division. The wood-rot fungus degraded wood cell walls by lytic *trans*-glycosylases (26). These results indicated a possible role of 1C2 in glycometabolism during birch wood decay by *P. betulinus*.

The 1C3 clone was identical to a polysaccharide deacetylase gene. This family of polysaccharide deacetylases includes Nod B (nodulation protein B from *Rhizobium*), which

is a chitoooligosaccharide deacetylase, and also includes chitin deacetylase from yeast, and endoxylanases which hydrolyse glucosidic bonds in xylan (15). These enzymes detected in both the intra- and extracellular fractions are requisite for degradation of plant structural and storage saccharides (25). So it may be inferred that polysaccharide deacetylase from *P. betulinus* may contribute to birch wood decay.

According to the result of the similarity analysis the 1D1 clone encodes a putative inositol monophosphatase (IMP) which is present widely in fungi, vertebrate and higher plants. IMP is a soluble protein that catalyzes the removal of a phosphate from inositol phosphate substrates, and is required for inositol synthesis from glucose 6-phosphate and for breakdown of inositol trisphosphate, a second messenger generated by the phosphatidylinositol signaling pathway (11). Its aminoacid sequence contains a conserved motif, which is also shared by several other proteins related to MPTASE (including products of fungal QaX and qutG, bacterial suhB and cysQ, and yeast hal2) (33). IMP has been implicated to part a number of processes involved in cellulose degradation (23). So the 1D1 was differentially expressed and could be involved in the cellulose degradation during birch wood decay by *P. betulinus*.

1D2 showed similarity with a glucoamylase-like protein with amylase activity, containing four subunits. It is thought that only the gamma subunit has enzymatic activity, whereas the other three subunits have a regulatory role (16). To our knowledge, it is not known how this type of protein participates in wood decay by fungi.

Conclusions

In this study, we found that 29 differentially expressed cDNA bands (~4.7%) were especially specific to cDNA samples

from the mycelia of *P. betulinus* inoculated wood samples. Twelve clones were isolated, cloned and sequenced. Seven of them were highly homologous to known genes. These results suggested that the process of decaying birch wood by *P. betulinus* is intricate, involving enzyme or protein changes.

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