Method To Identify Specific Alleles of a *Phanerochaete chrysosporium* Gene Encoding Lignin Peroxidase

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A method to identify and differentiate allelic variants of the gene encoding lignin peroxidase isozyme H8 is presented. The strategy involves amplifying a variable region of the gene's carboxy terminus by use of the polymerase chain reaction and then probing with allele-specific oligonucleotides.

Lignin depolymerization is catalyzed by extracellular peroxidases of the white rot basidiomycete *Phanerochaete chrysosporium* (11). Under nitrogen limitation, the dominant lignin peroxidase (LiP) isozyme of BKM-F-1767 is H8. Tien and Tu (19) cloned and sequenced a cDNA encoding H8, which was designated ML-1. Subsequently, corresponding genomic clones H8 and LPOA were sequenced (9, 17, 21).

It is now clear that the LiPs are encoded by a family of closely related genes, but the precise number and organization of genes are uncertain (5, 6, 16). The situation has been complicated by the existence of allelic variants, inconsistent nomenclature, and the wide spread use of two different *P. chrysosporium* strains, BKM-F-1767 and ME446.

Establishment of allelic relationships is made possible by analysis of single-basidiospore cultures, which are homokaryotic (1, 18). The segregation of specific alleles in homokaryons can be recognized by restriction site polymorphisms among alleles (7, 16). Thus, a cDNA clone, ML-4 (2), and its genomic equivalent, LiPA, have been shown to be allelic variants of LPOA (ML-1 and H8) (7).

We report here a rapid and convenient approach to identifying *P. chrysosporium* strains and specific alleles. Using this method, we show that the newly described LiP gene GLG3 (13), reportedly derived from BKM-F-1767, originated from ME446 or related strains. Our evidence strongly suggests that GLG3 is either identical or allelic to a previously described ME446 gene, LG1 (3).

*P. chrysosporium* BKM-F-1767 and ME446 were obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wis. Following fruiting (8), germinating single basidiospores were isolated from agar plates with a needle and a dissecting microscope. Genotypes of BKM-F-1767 single-basidiospore cultures 2 and 10 were previously established (7). Suitable DNA could be extracted from cultures by a variety of rapid protocols (10, 14).

The strategy to differentiate alleles involved polymerase chain reaction (PCR) amplification of genomic DNA followed by probing with allele-specific oligonucleotides. Primers A and B were selected to amplify a 260-bp carboxy terminus region of LPOA and LiPA alleles (Fig. 1). This region contains the last intron of the gene. Because the published sequence of LG1 (3) does not extend to the region of primer B, it was initially uncertain whether the primers would amplify LG1 and its allele. To facilitate cloning, primers also featured an EcoRI adaptor (Fig. 1).

All PCR reactions were performed in 100-μl mixtures with the following buffer: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.5 mM MgCl₂, 0.2 mM each dNTP, 150 pmol of each primer, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus). Oligonucleotide primers and probes were synthesized by the β-cyanoethyl phosphoramidite method on an Applied Biosystems 391 (Foster City, Calif.) without further purification. Approximately 20 ng of DNA template was subjected to an initial cycle of denaturation (6 min, 94°C), annealing (2 min, 54°C), and prolonged extension (40 min, 72°C) followed by 35 cycles of denaturation (1 min, 94°C), annealing (2 min, 54°C), and extension (5 min, 72°C). A final 15-min extension at 72°C was also included. Following amplification, 1- to 5-μl aliquots were electrophoresed on agarose gels containing 1% NuSieve agarose (FMC, Rockland, Maine) and 2% SeaKem GTG agarose (FMC). Gels were blotted for 1 to 2 h to Nytran (Schleicher & Schuell, Keene, N.H.) according to the manufacturer's recommendations and then UV cross-linked (UV Stratalinker 1800; Stratagene Inc., La Jolla, Calif.).

Minor modifications of the Southern blot protocols of Wallace and Miyada (20) were used. Blots were prehybridized for 1 h at 42°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.0015 M sodium citrate), 5× Denhardt's reagent, 0.1% sodium dodecyl sulfate (SDS) and 100 μg of denatured calf thymus DNA per ml. Hybridizations were at 42°C for approximately 2 h in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaPO₄ and 1 mM EDTA [pH 7.7]), 5× Denhardt's reagent, and 0.1% SDS. Probes were 32P end labeled with T4 polynucleotide kinase. Unincorporated label was not removed. Blots were washed in 6× SSC–0.1% SDS three times for 5 min at room temperature. A final 15-min wash was in the same buffer prequilibirated to 42°C. Blots were exposed to Kodak XAR film with amplifying screen for 2 to 12 h, depending upon desired band intensity. For reprobing, blots were stripped by boiling in 0.01× SSPE–1% SDS for 20 min.

PCR products were routinely subcloned by one of two methods. In the first, the DNA was digested with EcoRI and ligated into a dephosphorylated vector such as Bluescript KSII (Stratagene). More recently, we have employed pCR1000 (12), which does not require any modification or purification of the DNA. The double-stranded templates were sequenced by the dideoxy chain-termination method (15) by using standard primers.

Two 17-mer probes, differing by a single base pair (Fig. 1), were used to identify LPOA and LiPA (Fig. 2). The LPOA...
indicated in genomic basidiospore parental containing gene published sequence probes. LPOA probe hybridized parental dikaryotic gene (Fig. 3).

![Diagram](image1)

**FIG. 1.** Alignment of LiP genes showing PCR primers and probes. LPOA and LiPA have been identified as allelic variants of the gene encoding isozyme H8 in *P. chrysosporium* BKM-F-1767 (7). LG1 is a closely related clone from strain ME446 (3). The published sequence of LG1 does not extend to the primer B position. Primers A and B were 5'-GAATTCTGAATAGTG GCTGC-3' and 5'-GAATTCTCTAGA AGCGAATTCC-3', respectively. The underline identifies the EcoRI adapter. Introns and 3' untranslated regions are in lowercase letters.

![Diagram](image2)

**FIG. 2.** Southern hybridization of PCR products probed with LPOA (left) and LiPA (right) 17-mers (Fig. 1). Lanes contain PCR amplifications of a vector containing LPOA (lane 1), genomic DNA derived from dikaryotic parental strain BKM-F-1767 (lane 3), DNA derived from BKM-F-1767 single-basidiospore derivative 2 (lane 4), and BKM-F-1767 single-basidiospore 10 (lane 5). Sizes of PCR products are indicated in margin.

![Diagram](image3)

**FIG. 3.** Southern hybridization of PCR products probed with LG1 (left) and LiPA (right) 17-mers (Fig. 1). Lanes contain PCR amplifications of plasmid pUGL3 containing genomic clone GLG3 (lane 1), genomic DNA derived from dikaryotic parental strain ME446 (lane 2), ME446 single-basidiospore derivative 1 (lane 3), and ME446 single-basidiospore 8 (lane 4). Sizes of PCR products are indicated in margin.

culture 8, hybridized to the LG1-specific probe. Interestingly, the LiPA-specific probe also hybridized to the parental DNA (lane 2) and to ME446 single-basidiospore culture 8. These results suggest that the allelic variant of LG1 in ME446 lacks the 9-bp deletion (Fig. 1). Subsequent cloning and sequencing of the PCR products of cultures 1 and 8 confirmed this (data not shown). The nucleotide sequence of the last intron of culture 8 was identical to LiPA except for one mismatch at position 152 (Fig. 1). The LG1 probe never hybridized to any BKM-F-1767 DNA. This latter observation was unexpected because the nucleotide sequence of GLG3 (13), a genomic LiP clone reportedly derived from BKM-F-1767, aligned perfectly with the LG1 probe. These hybridization patterns strongly suggest that GLG3 was not derived from BKM-F-1767.

Several lines of evidence suggest that the amplified product of ME446 single-basidiospore culture 1 is identical to GLG3 and LG1. The LG1 probe, although never hybridizing to BKM-F-1767 DNA, did hybridize to parental ME446 (Fig. 3, lane 2), single-basidiospore culture 1 (Fig. 3, lane 3), and amplified plasmid pUGL3 (Fig. 3, lane 1). The latter plasmid contains the genomic GLG3 clone and was obtained from C. A. Reddy (Michigan State University). In addition, our analysis of pUGL3 showed the presence of two 11-bp sequencing errors in the 3' untranslated region immediately adjacent to the putative translational stop codon. When these corrections are considered, the nucleotide sequence of GLG3 is 97.9 and 99.2% identical to LPOA and LG1, respectively. Finally, the nucleotide sequence of the PCR product of culture 1 is identical to the corrected GLG3 sequence. Thus, GLG3 is identical to ME446 clone LG1.

The PCR approach described here allows the rapid identification of specific LiP genes and alleles. The method will facilitate a variety of investigations, including genetic analyses of homokaryotic segregants and identification and differentiation of *P. chrysosporium* strains. The precise identification of genes within strains is an absolute prerequisite for studies of the transcriptional regulation of closely related LiP genes. The method may also be useful for taxonomic investigations of this diverse species (4). Refinements and modifications of the technique may be appropriate under certain circumstances. For example, dot blots could be substituted for Southern blots of gels, and nonradioactive oligonucleotide probes could be used.
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