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Expression of *Phanerochaete chrysosporium* Genes Encoding Lignin Peroxidases, Manganese Peroxidases, and Glyoxal Oxidase in Wood

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Expression of *Phanerochaete chrysosporium* genes encoding ligninolytic enzymes was assessed in wood. Poly(A) RNA was extracted from colonized wood chips by magnetic capture, and specific transcripts were quantified by competitive reverse transcriptase PCR. mRNA levels varied substantially among lignin peroxidase genes, and transcript patterns were dramatically different from those in previous studies with defined media.

Lignin depolymerization is catalyzed by extracellular enzymes of white rot basidiomycetes such as *Phanerochaete chrysosporium*. Major components of this system include lignin peroxidases (LiPs), manganese-dependent lignin peroxidases (MnPs), and a peroxide-generating enzyme, glyoxal oxidase (GLOX) (for review, see references 6, 11, and 17). Under nutrient limitation in defined media, multiple peroxidase and GLOX isozymes are secreted.

The peroxidases of *P. chrysosporium* are encoded by families of structurally related genes. Ten LiP genes, designated *lipA* through *lipJ*, have been characterized and shown to be distributed on three linkage groups (reviewed in reference 12). The three known MnP genes (*mnp* genes) are unlinked to each other or to any LiP genes (reference 28 and unpublished data). In contrast, GLOX is encoded by a single gene (*glx*) with two alleles (20, 23). The precise roles and interactions of these genes in lignin degradation and in commercial processes such as biomechanical pulping (for review, see reference 24) are poorly understood.

Numerous studies have demonstrated differential regulation of LiP and MnP genes in response to culture conditions. Northern blots showed *lipD* transcripts dominating in carbon-starved cultures (18) and in defined media supplemented with balled-mill straw (19). In contrast, *lipA* transcripts were relatively more abundant in nitrogen-limited media (18). Nuclease protection assays identified *lipE* as the major transcript in both carbon- and nitrogen-starved cultures (30). Quantitative reverse transcriptase-mediated PCR (RT-PCR) techniques largely confirmed Northern blots and also showed dramatic upregulation of *lipC* and *lipJ* under nitrogen starvation (31). All LiP gene transcripts except *lipF* were detected in anthracene-contaminated soil cultures (3). The MnP genes of *P. chrysosporium* exhibit complex regulation by nutrient limitation (15, 29), Mn concentration (7, 9, 14), culture agitation, heat shock (8), H_2O_2 concentration, and other chemical stresses (25). *mnp3* appears not to be regulated by Mn. In contrast, *mnp1* and *mnp2* respond strongly to Mn and are differentially regulated in response to culture agitation (15). The three MnP genes are coordinately transcribed in soil cultures (4). Nothing is known of the regulation of *P. chrysosporium* peroxidase genes in woody tissue, the natural substrate.

To assess transcript levels of all known LiP, MnP, and GLOX genes in *P. chrysosporium*-colonized wood, 2.5 kg of aspen wood chips was steam sterilized and inoculated by standard biomechanical pulping methods (1) (reviewed in reference 2). Poly(A) RNA was extracted from 10-g samples as described elsewhere (32), with minor modifications. Specifically, the initial extract buffer was squeezed through Miracloth (Calbiochem, Inc., La Jolla, Calif.) filters, and following incubation with Dynabeads oligo$(dT)_{25}$ (Dynal, Great Neck, N.Y.), the hybridization buffer was twice extracted with a model MPC-1 magnetic concentrator. Poly(A) RNA levels were too low to accurately quantify (<1 μg/10 g), but yields were adequate for a minimum of 600 separate RT-PCRs. The competitive RT-PCR protocol was adapted from the work of Gilliland et al. (16) with gene-specific primers (Table 1). Competitive templates, in the form of full-length genomic subclones, were added to 50-μl PCR mixtures as 10-fold serial dilutions ranging from 10 ng to 0.1 fg. Preliminary experiments quantifying *lipA*, *lipC*, *lipE*, and *lipF* transcripts with various amounts of poly(A) template in RT-PCRs showed no evidence for RT inhibition (10).

PCR products were size fractionated on 1.5% agarose gels and ethidium bromide stained, and the image was recorded with a Foto/Analyst digital camera (Fotodyne, Inc., Hartland, Wis.). The image was digitized with NIH Image software (version 1.61). Linear regressions were determined by plotting ratios of genomic competitor to cDNA target against the concentration of competitive template. Adjusting for length differences, equivalence points were determined on linear regressions where the ratios were 1.5 for *lip* genes, 1.3 for *mnp* genes, and 1.19 for *glx*. Results were expressed in picograms of cDNA (Fig. 1). Independent analysis for *lipC* and *mnp2* transcript levels in separate wood chip cultures varied less than 12%.

Differences in transcript levels ranged up to 10,000 fold (Fig. 2), and transcript patterns in aspen were unlike the patterns previously observed in defined media or in soil cultures. Transcripts of *lipF*, absent in soil cultures, were abundant. *lipD* and *lipE*, major transcripts in soil and defined media, ranked lowest among LiP gene transcripts in aspen. Transcripts of *lipI*, rep-

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TABLE 1. Competitive PCR primers

Gene[a]	5′ primer	3′ primer
lipA	TCCATCGCAATTTCGCCC	ACACGGTTGATGATTTGG
lipB	GCTATTGCCATCTCTCCT	ACACGAGCGATGATCTGG
lipC	GCCATCGCTATCTCTCCC	ACACGGTCGATGATTTGG
lipD	TCCATCGCTATCTCGCCC	ATGCGAGCGAGAACCTGA
lipE	TCCATCGCCATCTCGCCC	ACGCGGGCGATGATCTGG
lipF	TGCCCTTGAGTCTCAAGG	ACGCGAGAGATGATCTGG
lipG	TCGATCGCCATCTCGCCC	ACACGCTCGATGAGCTGG
lipH	GCAATTGCCATCTCGCCC	ACACGGTTAATGAGCTGG
lipI	TCTATCGCTATCTCTCCC	ACACGGCTGATGATTTGA
lipJ	GCCATCGCGATCTCTCCC	ATCCGAGCCAGGATCTGA
mnp1	CCGACGGCACCCGCGTCAGC	CGAGCGGGAGCGGCGACGCC
mnp2	CAGACGGTACCCGCGTCACC	AGTGGGAGCGGCGACATCAC
mnp3	CCGACGGTACCAAGGTCAAC	AGCGGCAGCGGCGACGCGAC
glx	TCACACCTTCGCTCTACACG	TATTTACTCCAGGGTCGGCG

[a] Excluding *lipJ*, LiP gene primers were previously described (3). MnP gene primers were described previously (4), but gene designations have been revised to conform to the work of Gettemy et al. (15).

resented by a single functional allele (*lipI1*) in dikaryotic strain BKM-F-1767, were at high levels relative to those in defined media (31). (The alternative allele, *lipI2*, is transcriptionally inactive due to insertion of a repetitive element [13].) Relative

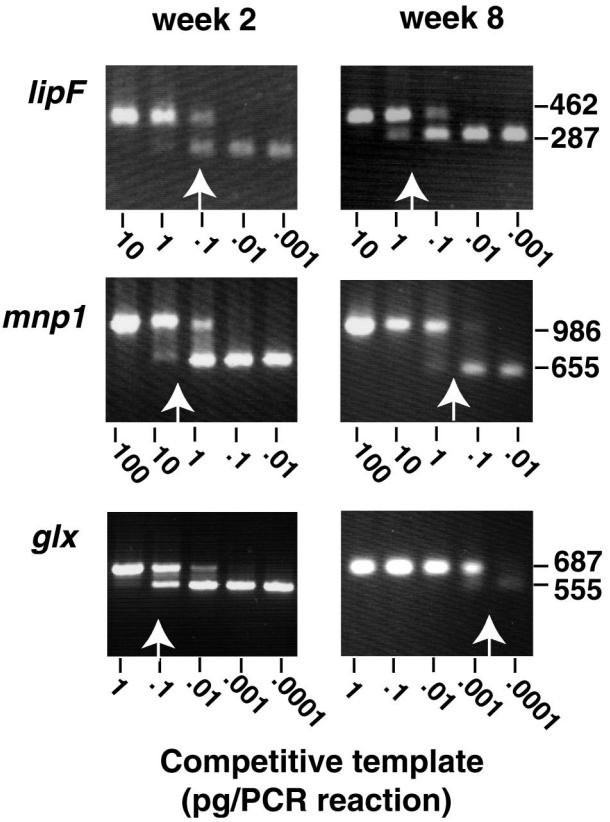

FIG. 1. Competitive PCRs comparing transcripts of three genes in samples collected after 2 and 8 weeks of incubation. Vertical arrows represent approximate equivalence points. Digitized images were acquired and labeled with Adobe Photoshop 3.0 and Illustrator 7.0, respectively. Numbers at right indicate molecular size in base pairs.

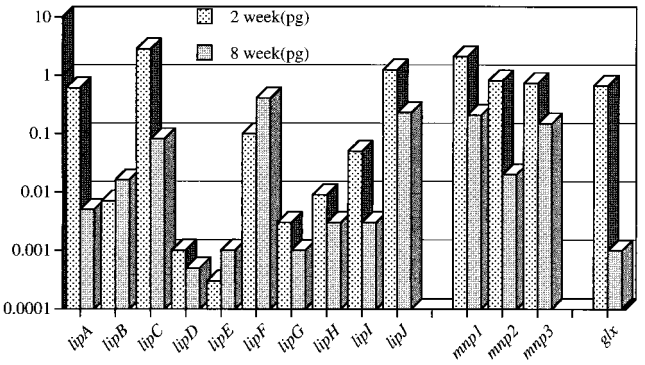

FIG. 2. Comparison of transcript levels of all known peroxidase and GLOX genes in aspen wood chip cultures after incubation for 2 or 8 weeks. Results are expressed as picograms of cDNA. The nomenclature for *lip* and *mnp* gene designations follows the work of Gaskell et al. (12) and Gettemy et al. (15), respectively.

to *lip* genes, the *mnp* genes showed less difference in transcript levels.

The identification of *glx* transcripts in wood was consistent with a close physiological connection between extracellular peroxidases and GLOX (21, 22). The simultaneous detection of *lip* and *glx* transcripts was reported in defined media (20, 23) and in soil cultures (3).

Transcript levels generally declined by 8 weeks of incubation, although it is unclear whether transcription was reduced or whether mRNA was partially degraded. *lipA* and *glx* transcripts decreased more than 100-fold, while *lipB*, *lipE*, and *lipF* transcripts increased 3- to 4-fold. Temporal shifts in transcription have been observed in defined media (3, 5, 25).

No clear relationship between genomic organization and transcription emerges from these and previous results. Within the two LiP gene clusters (*lipA*, *lipB*, *lipC*, *lipE* and *lipI*, *lipG*, *lipH*, *lipJ*), no patterns are evident. The unlinked LiP genes, *lipD* and *lipF*, show patterns very different from those of one another and from those of most other *lip* genes. In comparing all genes on all substrates, *lipD* and *lipE* transcript patterns are most alike, although the *lipD* transcript levels are consistently 5- to 10-fold higher than those of *lipE*.

The substantial differences in transcript levels probably reflect enzyme activity in aspen, as has been demonstrated in defined media (5, 26, 27) and in soil cultures (3, 4). Thus, genes previously shown to be highly expressed under a wide range of cultural conditions, such as *lipD* and *lipE* (18, 19, 30), are unlikely to play a major role in biopulping performance on aspen. It remains to be determined if these genes are differentially regulated under other conditions (e.g., wood species, temperature, and moisture).

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