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Differential Regulation of *mnp2*, a New Manganese Peroxidase-Encoding Gene from the Ligninolytic Fungus *Trametes versicolor* PRL 572

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A peroxidase-encoding gene, *mnp2*, and its corresponding cDNA were characterized from the white-rot basidiomycete *Trametes versicolor* PRL 572. We used quantitative reverse transcriptase-mediated PCR to identify *mnp2* transcripts in nutrient-limited stationary cultures. Although *mnp2* lacks upstream metal response elements (MREs), addition of $MnSO_4$ to cultures increased *mnp2* transcript levels 250-fold. In contrast, transcript levels of an MRE-containing gene of *T. versicolor*, *mnp1*, increased only eightfold under the same conditions. Thus, the manganese peroxidase genes in T. versicolor are differentially regulated, and upstream MREs are not necessarily involved. Our results support the hypothesis that fungal and plant peroxidases arose through an ancient duplication and folding of two structural domains, since we found the *mnp1* and *mnp2* polypeptides to have internal homology.

Peroxidases catalyze one-electron oxidation of a wide range of organic and inorganic substrates. Enzymes from plants, fungi, and bacteria are evolutionarily related and fundamentally different from the heme peroxidases of animal origin (29). The plant peroxidase superfamily contains two families of ligninolytic enzymes, lignin peroxidase (Lip) and manganese peroxidase (MnP) (29). These extracellular peroxidases have been isolated from various white-rot basidiomycetes, such as *Trametes (Coriolus) versicolor* (12, 15, 18) and *Phanerochaete chrysosporium* (10). Their biological function is thought to be oxidative lignin degradation in rotting wood (7).

T. versicolor produces multiple Lip and MnP isozymes, which are encoded by families of structurally related genes (12, 14). Several peroxidase-like genomic clones have been sequenced (2, 13, 14, 16, 19, 20), although some sequences are presumed to be allelic variants (14). Partial amino acid sequences have been determined for *T. versicolor* isozymes designated LiP12, LiP7, and MnP2 (15), which are encoded by *lip1/lip4*, *lip6*, and *mnp1*, respectively (13, 14). The role and interactions of *T. versicolor* peroxidases are poorly understood, and their transcriptional regulation has not been investigated.

In this paper we report the genomic and cDNA sequences for a second *T. versicolor* MnP gene, *mnp2*. By comparative analysis with available sequences, mnp2 was placed in the MnP subfamily. We show that transcript levels of *mnp1* and *mnp2* are regulated by medium composition.

We isolated mnp2 from a previously constructed l GEM-11 (Promega Corp., Madison, Wis.) genomic library (19) of *T. versicolor* PRL 572, assembled from partially *Sau*3AI-cleaved genomic DNA. The probe TvM2 (36-mer, 5'-GACGACAT CCAGAAGAACCTCTTCGACGGCGGCGAG-3') was designed based on N-terminal-amino-acid sequence information obtained from the MnP2 isoform and corresponds to amino acid residues 23 to 34 (14, 15). Several positive l clones were purified and a 7.3-kb *Xho*I fragment was subcloned into plasmid vector pGEM-7Zf(+) (Promega). Screening of the library and confirmation of clones were performed by standard procedures (23) for plaque or colony hybridization and Southern blotting (Hybond-N+; Amersham Pharmacia Biotech., Piscataway, N.J.). A cDNA corresponding to mnp2 was amplified by reverse transcriptase PCR with *Pfu* polymerase (Stratagene Corp., La Jolla, Calif.) from total RNA using primers 867 (5'-CCATCCCTCAAAACCTCAGG-3') and 899 (5'-ACTC CCACCTCAAGCTTAAG-3'). The 1,132-bp cDNA was subcloned directly into pGEM-T Easy (Promega). Genomic and cDNA clones were sequenced by the dideoxy termination method (24). Multiple alignments of predicted amino acid sequences showed that the mnp2 sequence was most closely related to *T. versicolor mnp1* (GenBank/EMBL accession number 230668) (78% identical in amino acid sequence), although the intron-exon arrangement is different between the two genes (Fig. 1A).

Peroxidases have 10 a-helical regions (A to J) that are folded into distal and proximal domains that surround and commonly coordinate the prosthetic heme molecule through the distal and proximal histidines (30). Fungal and plant peroxidases may have originated from a gene duplication that occurred through the folding of the two structural domains (30). The *mnp2* and *mnp1* sequences have internal homology in the predicted proteins that is consistent with this hypothesis. Alignment of the amino acid sequences encoded by *mnp1* exons 2 and 3 and exon 4 (Fig. 1B) shows a spatial conservation and 23% sequence identity (35% homology). Helices A to I, as predicted by Sundaramoorthy et al. (27), are roughly aligned, as are several residues proposed to bind Ca^{2+} (Fig. 1B). Furthermore, the second exon of *mnp2* and the fourth exon of *mnp1* (Fig. 1C) show 18% sequence identity (28% homology),

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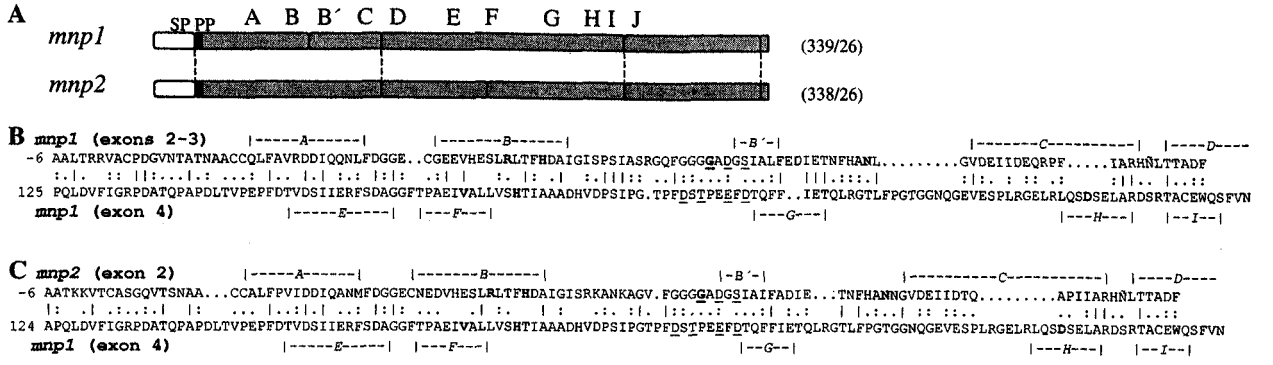

FIG. 1. Analysis of domain structures from amino acid sequences predicted from specific exons in the *T. versicolor mnp1* (14) and *mnp2* (this study) genes. (A) Schematic presentation of the relation between exons predicted from *mnp1* and *mnp2*. Dashed lines indicate related intron-exon junctions. Unshaded areas show the exons encoding signal peptides (SP). Lightly shaded and black areas indicate mature peptides and putative propeptides (PP), respectively. Numbers in parentheses are the expected numbers of amino acid residues in the mature polypeptide and in the signal and propeptide. The predicted helical conformation (A to J) is in accordance with reference 27. (B) Alignment of amino acid sequences predicted from exons 2 and 3 and exon 4 in *mnp1*. (C) Alignment of amino acid sequences predicted from exon 2 in *mnp2* and exon 4 in *mnp1*. Residues in boldface are the 10 invariant amino acid residues within the plant peroxidase superfamily (30), and underlined residues are proposed to bind Ca^{2+} (22, 27). Alignment (GAP; Genetics Computer Group, Madison, Wis.) displays identities (|) and similarities (: or .) between sequences. Similarity reflects evolutionary relationship between codons rather than chemical characteristics of side chains.

suggesting that they may be homologous to an undisrupted ancestral domain.

We cultured *T. versicolor* in four different types of liquid media. M2 medium has been described (12, 17) and is limited in both carbon (11 mM glucose) and nitrogen (5 mM diammonium tartrate). M1 medium is like M2 but with fivefold-higher molar amounts of carbon and nitrogen. M3 medium is like M1 but with a 3 mM veratryl alcohol supplement, and M4 is identical to M3 but with 200 μM $MnSO_4$ (12). Liquid cultures were made in 250-ml Erlenmeyer flasks containing 37 ml of medium and inoculated with 750 μl of homogenized mycelium prepared as previously described (12). Duplicate cultures were set up for each medium (M1 to M4) and each time point (4 to 12 days). Flasks were incubated stationary at 27°C. Extracellular media and the mycelium were separated by filtering through Miracloth (Calbiochem-Novabiochem, La Jolla, Calif.). The mycelium was frozen in liquid nitrogen and ground in a mortar with a pestle precooled in dry ice and stored at −80°C. The extracellular filtrate was placed on ice, and the enzyme activity was determined. The MnP activity was measured as Mn^{2+} oxidation (12); each sample was measured twice, and activities are presented as mean change in absorbance at 300 nm (DA) per minute per milliliter. As predicted, there was a significant increase in extracellular MnP enzyme activity after addition of Mn^{2+} (M4) (Fig. 2A), an activation that has been observed in several systems, such as *T. versicolor* (12, 28) and *P. chrysosporium* (6, 21). MnP activity could not be detected under any other culture conditions tested (M1 to M3) (Fig. 2A).

Relative transcript levels of *mnp2* and *mnp1* were estimated by reverse transcription coupled to competitive PCR (RT-cPCR) (9). The technique involves oligo(dT)-primed RT followed by replicate PCRs containing 10-fold serial dilutions of competitive template. In our experiments, the competitors were intron-containing, genomic clones. Following PCR amplification, the cDNA target and genomic product were size fractionated on agarose gels. The dilution at which cDNA and larger genome products are equivalent is an indirect measure of transcript concentration. The technique is well suited for quantifying closely related peroxidases, although reproducibility ranges from an average standard deviation of 12 to 57% when 10-fold dilutions are used. Differences beyond twofold are highly significant (4, 11, 25, 26).

Total RNA was isolated from approximately 100 mg (wet weight) of mycelium with an RNeasy plant minikit (Qiagen Inc., Valencia, Calif.) and eluted in 60 μl of H_2O. With minor modifications, the RT reactions followed protocols recommended for Superscript II reverse transcriptase (Life Technologies, Rockville, Md.) and contained 2 μg of total RNA, 45 pmol of oligo$(dT)_{15}$, and 30 U of RNasin RNase inhibitor (Promega). The 50-μ1 cPCR mixtures were cycled as described previously (26) and contained 1 μ1 of the diluted first-strand product, 5% dimethyl sulfoxide, 15 pmol of each of two template-specific primers, and the competitive template. Primers 867 and 899 were used for *mnp2* amplifications while primers 866 (5'-CGTCTATCCTCCTCCTCATA-3') and 908 (5'-CTACAGCGTCATITACGACG-3') were used to amplify *mnp1*. Full-length genomic copies of *mnp1* and *mnp2* subcloned into pGEM-T Easy (Promega) served as competitive templates, and these were added to PCRs as 10-fold serial dilutions ranging from 10^{-6} to 10 ng of DNA. The control PCRs received no competitive template. To ensure the specificity of PCR amplifications, control reaction cDNAs were partially purified and sequenced directly by using Big Dye termination reactions (Applied Biosystems, Foster City, Calif.) run on an ABI 377 automated sequencer (Applied Biosystems).

mnp2 was found to be differentially regulated in response to culture conditions (Fig. 2B and C). *mnp2* cDNA levels rose from approximately 4×10^{-5} to 1×10^{-2} ng (250-fold) when M3 medium was supplemented with Mn^{2+} but showed no response to increased amounts of carbon and nitrogen or addition of veratryl alcohol. In contrast, *mnp1* transcript levels increased only 8-fold, from 5×10^{-5} to 4×10^{-4} ng, in the Same cultures (Fig. 2C). In samples harvested on day 6, levels of *mnp2* and *mnp1* transcripts were similar, whereas the dif-

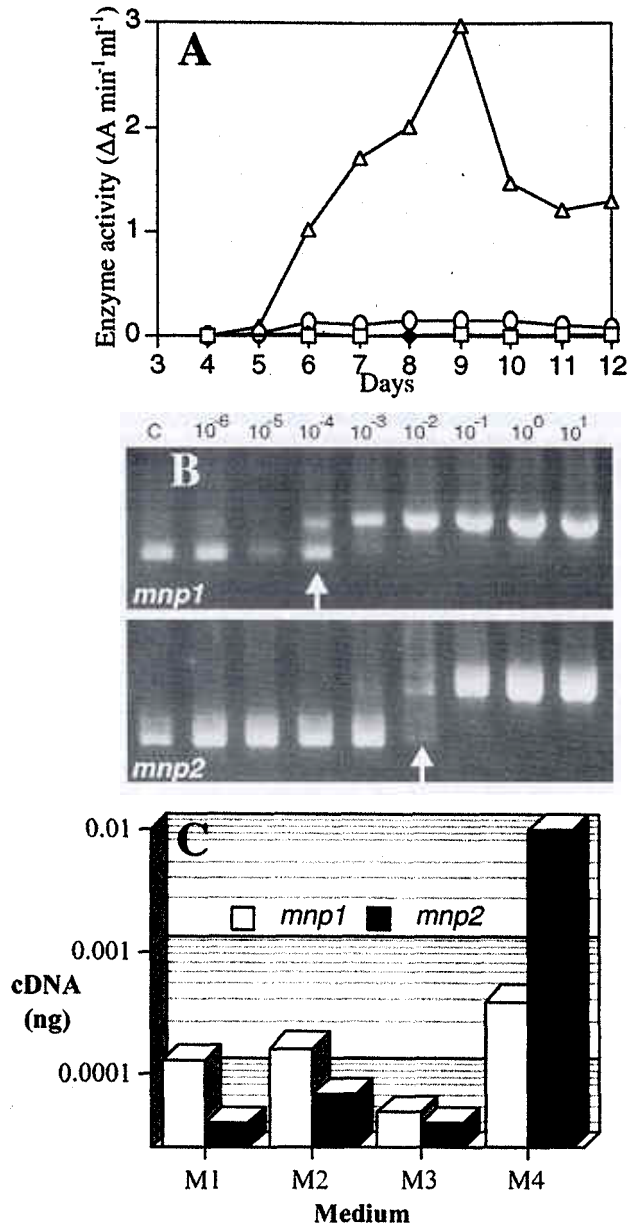

FIG. 2. Extracellular MnP activities and gene-specific transcript levels measured under different growth conditions. (A) Extracellular MnP activity in *T. versicolor* liquid cultures during 12 days of incubation in M1 (□), M2 (◆), M3 (○), and M4 (△). Enzyme activities are means for duplicate cultures. (B) RT-cPCR for the quantification of *T. versicolor mnp1* and *mnp2* transcript levels after 9 days of growth in M4. Lanes were loaded with PCR mixtures that received increasing amounts (indicated in nanograms of plasmid DNA) of competitive template, except for the control (C). Primers were chosen for full-length amplification of either cDNA (1,132 bp) or the competitive genomic templates (1,432 or 1,450 bp). Arrows indicate the approximate dilution point where concentrations of the cDNA and competitive product are equivalent. (C) Compilation of *mnp1* and *mnp2* transcript levels after 9 days of growth in either M1, M2, M3 or M4, as determined by cPCR.

ference observed on day 9 (Fig. 2B and C) was even more pronounced on day 12 (data not shown).

Quantitative transcript analyses of the three known *P. chrysosporium* MnP genes generally show coordinate regulation (3, 11). Putative metal response elements (MREs) have been have been identified upstream of *P. chrysosporium mnpl* and *mnp2*, and their transcript levels increase substantially in response to Mn^{2+} supplementation of low-nitrogen media (8). Transcript levels of a *P. chrysosporium* gene lacking paired MREs, *mnp3*, are not influenced by addition of Mn^{2+} (5, 6, 8). Taken together, these observations suggest an important role for MREs in transcriptional regulation of *P. chrysosporium* MnP genes (1, 8). In contrast, *T. versicolor* MnP regulation appears not to involve MREs. Putative MREs have been identified in *T. versicolor mnp1* (14) but not in *mnp2*. Consequently, the positive Mn^{2+} regulation of *mnp2* must be governed by other means.

Differential regulation of *T. versicolor* and *P. chrysosporium* MnP genes in response to culture conditions is consistent with an important role for the gene families in adapting to environmental conditions. Factors altering MnP transcript levels include Mn^{2+}, heat shock, and chemical stress (10), but the controlling mechanisms remain unclear. The recently sequenced *P. chlysosporium* genome (www.jgi.doe.gov/programs/whiterot/whiterot_mainpage.html) reveals at least one additional MRE-containing *mnp* gene (data not shown). Analysis of this and other MnP genes may help explain the gene multiplicity often observed among lignin-degrading fungi.

Nucleotide sequence accession number. The genomic and cDNA clones identified in this study were assigned GenBank/EMBL accession numbers 254279 and AF10215, respectively.

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