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Note

Cloning and Transcriptional Analysis of the Gene Encoding 5-Aminolevulinic Acid Synthase of the White-Rot Fungus *Phanerochaete sordida* YK-624

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In this study, we cloned the gene encoding 5-aminolevulinic acid synthase (ALAS) from the hyper-lignin-degrading fungus *Phanerochaete sordida* YK-624. The deduced amino acid sequence showed highest identity (93.0%) to ALAS of *P. chrysosporium*. Expression of the gene encoding ALAS, which we named *aas*, corresponded temporally with the expression and activity of manganese peroxidase.

Key words: *Phanerochaete sordida* YK-624; 5-aminolevulinic acid synthase; gene cloning; transcriptional analysis

Many white-rot fungi produce multiple extracellular ligninolytic enzymes to degrade lignin, a heterogeneous, random phenylpropanoid polymer. These enzymes are divided into four major families: laccase,[1)] manganese peroxidase (MnP),[1)] lignin peroxidase (LiP),[1)] and versatile peroxidase (VP).[2,3)] The active sites of MnP, LiP, and VP contain heme, which participates in the oxidation of substrates.[4)] Intracellular cytochrome P450s, which mediate the oxygenation reactions of aromatic compounds such as lignin degradation products,[5–7)] are also hemeproteins. Thus, the biosynthesis of heme is especially important in the biodegradation of lignin by white-rot fungi.

The biosynthesis of heme in all living cells occurs through several steps, in which 5-aminolevulinic acid (ALA) is the first committed intermediate. Two alternative routes for the formation of ALA have been proposed: one in which the condensation of succinyl CoA and glycine is catalyzed by ALA synthase (ALAS, EC 2.3.1.37) in the mitochondria, and the second, called the 5-carbon route, which occurs in the stroma of plastids.[8)] In the white-rot fungus *Phanerochaete chrysosporium*, with a 30-million-bp genome sequenced by the whole shotgun approach,[9)] both the expression of the ALAS-encoding gene and the production of MnP is increased by vanillin,[10)] suggesting that ALAS participates in the biosynthesis of heme by white-rot fungi. In the white-rot fungus *P. sordida* YK-624, which was isolated from rotten wood and has much higher ligninolytic activity and ligninolytic selectivity than *P. chrysosporium* or *Trametes versicolor*,[11)] the major extracellular ligninolytic enzymes are MnP[11,12)] and LiP.[13)] Cloning of the gene encoding ALAS from white-rot fungi is thus necessary to generate strains that overproduce these ligninolytic peroxidases. Although the complete genome sequence is not available, we cloned the gene encoding ALAS from *P. sordida* YK-624 and examined its transcription.

We cloned the cDNA and the genomic DNA encoding ALAS from *P. sordida* YK-624 by a series of PCRs. *P. sordida* YK-624 was cultured for 5 d in nitrogen-limited liquid Kirk medium.[14)] Total RNA and genomic DNA were isolated from a piece of *P. sordida* YK-624 mycelium using an RNeasy Plant Mini kit (Qiagen, Valencia, CA) and ISOPLANT II (Nippon Gene, Tokyo) respectively. The conserved region of the ALAS-encoding gene was amplified using degenerate forward primer ALASdF (corresponding to amino acid residues YRYFNNI) and degenerate reverse primer ALASdR (corresponding to PKIIAFE) with a Primescript RT-PCR kit (TaKaRa Bio, Shiga, Japan). The amplified cDNA fragment was cloned and sequenced. The 3′-coding region of the gene was cloned by 3′-rapid amplification of cDNA ends (RACE) using a 3′-Full RACE core set (Takara Bio) and gene-specific primer ALAS3′F. The 5′-coding region of this gene was cloned from genomic DNA by an inverse PCR method[15)] with primer sets InverseF1-InverseR1 and InverseF2-InverseR2, which were designed on the basis of the 3′-RACE results. The genomic DNA was digested with *Kas* I (New England Biolabs, Ipswich, MA) and self-ligated with T4 DNA ligase (Nippon Gene) to give circular DNA products. Inverse PCR resulted in a partial 5′-coding sequence. To obtain the full-length cDNA for the ALAS-encoding gene, the 5′-coding region was cloned by 5′-RACE using a 5′-Full RACE core set (Takara Bio), with a 5′-phosphorylated primer and two nested primer sets, corresponding to inverse PCR fragments ALASpR and ALAS5′F1-ALAS5′R1 and ALAS5′F2-ALAS5′R2.

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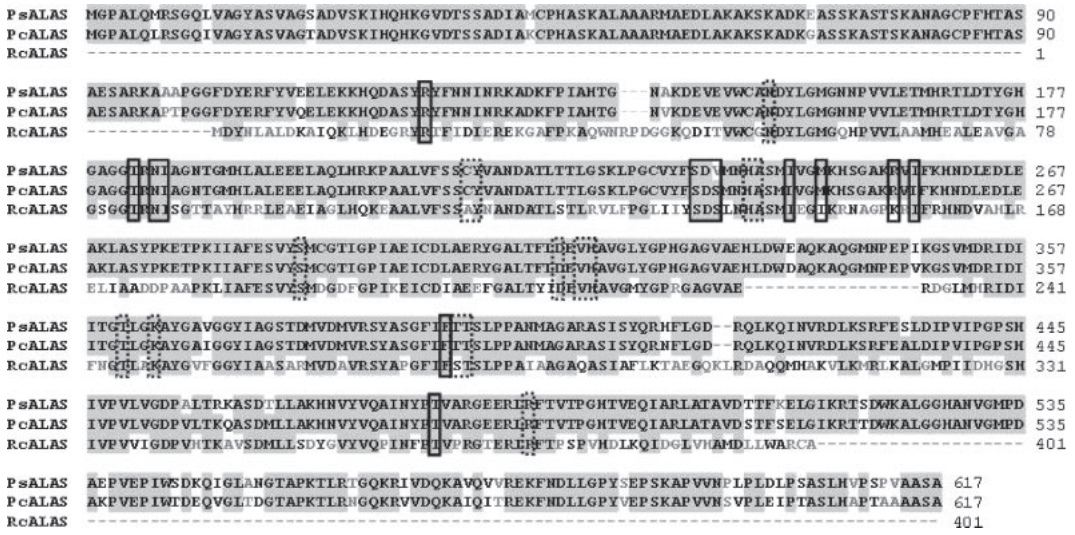

Fig. 1. Amino Acid Sequence Alignment of PsALAS, PcALAS, and RcALAS.

Identical and similar residues are shown in black with and without gray shading. All other residues are shown in gray. Solid-line boxes indicate predicted succinyl-coenzyme A binding residues. Dotted-line boxes indicate predicted glycine-bound pyridoxal 5′-phosphate binding residues.

Table 1. Oligonucleotides Used as Primers in This Study

Primer name	Nucleotide sequence (5′-sequence-3′)
ALASdF	TAYMGNTAYTTYAAYAAYAT
ALASdR	TCRAANGCDATDATYTTNGG
ALAS3′F	CAAGCACAACGATCTCGAAG
InverseF1	TCGAAGACCTCGAAGCCAAG
InverseR1	AAGATAGTCGTTGGCACACC
InverseF2	TCGCATCCTATCCCAAGGAG
InverseR2	ACACCTCCACCTCATCCTTC
ALAS5′F1	CCTACCGCTACTTCAACAAC
ALAS5′R1	TGGTGCTTCTTCTCCAACTC
ALAS5′F2	CAACGACTATCTTGGCATGG
ALAS5′R2	AGAACCGCTCGTAGTCAAAG
ALASpR*	TCTCCAGTACCACTG
ALASF1	CAAAGTTCAAGTCCTCGTGC
ALASR1	CTTGCACGTACTACGACTTC
ALASF2	AAGGATGAGGTGGAGGTGTG
ALASR2	GAGTGCCAGTGATGATGTCG
MnP4F	ACGCACAACACTATCAGTGC
MnP4R	GAATGGTGTAGAGTCGAACG
ActinF	AGCACGGTATCGTCACCAAC
ActinR	AGCGAAACCCTCGTAGATGG

Abbreviations: N = A, T, G, or C; D = G, A, or T; R = A or G; M = A or C; Y = C or T

*The 5′ end was phosphorylated.

Primer set ALASF1-ALASR1, designed based on results of 3′-RACE and 5′-RACE, was used to determine the sequence of the full-length cDNA and genomic DNA encoding ALAS of *P. sordida* YK-624, and the gene obtained was named *aas*.

The full-length cDNA fragment for *aas* was 2,203 bp and the genomic DNA fragment was 2,321 bp (gene accession no. AB525687), and the genomic DNA had five exons and four introns. The nucleotide sequence of the cDNA was entirely identical with that of the exons in the genomic DNA. A comparison of the deduced amino acid sequences for ALAS of several organisms indicated that Aas (PsALAS) had highest identity to ALAS of *P. chrysosporium* (PcALAS, 93.0%). X-Ray crystallography identified the substrate-binding sites of the structure of ALAS from *Rhodobacter capsulatus*

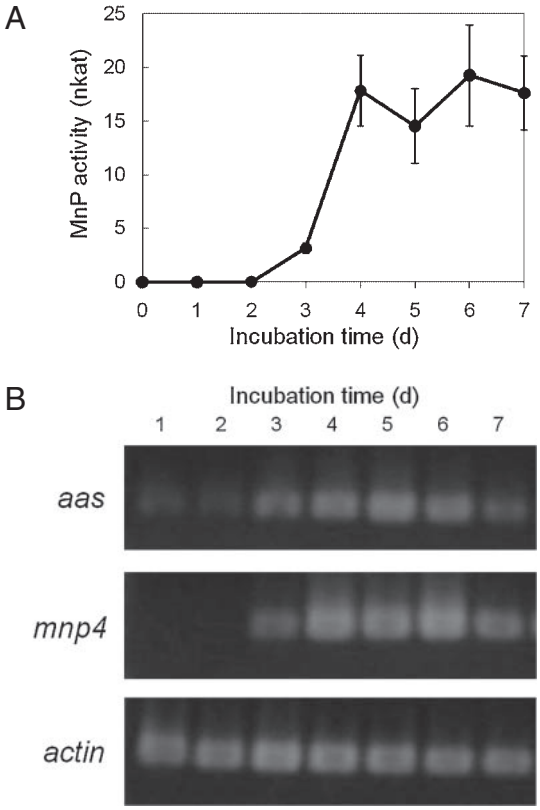

Fig. 2. Production of MnP and Expression of *aas* and *mnp4* by *P. sordida* YK-624.

A, MnP activity in the extracellular culture fluid. Bars indicate standard deviation. B, RT-PCR analysis of *aas* and *mnp4*. The cDNA encoding *actin* was amplified as a positive control.

(RcALAS), a nonsulfur photosynthetic bacterium.[16] Amino acid sequence alignment of PsALAS, PcALAS, and RcALAS (Fig. 1) indicated that many of the substrate-binding residues are conserved in PsALAS, although the sequence identity between PsALAS and RcALAS was 34.2%.

Next we analyzed the transcription of *aas*. *P. sordida* YK-624 was grown on 9-cm PDA plates for 3 d at 30 °C.

Two 9-mm mycelial disks were punched from the growing edge of the mycelium, and were added to a 100-mL Erlenmeyer flask containing 10 mL of nitrogen-limited Kirk medium. MnP and LiP activities were measured each day (Fig. 2A), and mycelia were collected and stored at −80 °C. Total RNA for each mycelium was isolated using an RNeasy Plant Mini kit. RT-PCR was performed using 200 ng of total RNA with a PrimeScript RT-PCR Kit and gene-specific primer sets ALASF2-ALASR2, MnP4F-MnP4R, and ActinF-ActinR (Fig. 2B). MnP activity was detected after 3 d of cultivation, and a high level of MnP activity was detected after 4 to 7 d. There was little LiP activity throughout the incubation period (data not shown). The N-terminal amino acid sequence analysis indicated that the main isoform of MnP in these cultures was Mnp4 (gene accession no. AB585997, *mnp4*). Hence, we used gene-specific prime set MnP4F-MnP4R for RT-PCR of MnP. RT-PCR indicated high levels of *aas* expression at 3 to 7 d. Similar results were found for *mnp4*. This suggests that the expression of *aas* correlates with that of *mnp*. In further studies, we intend to generate a strain of *P. sordida* YK-624 with simultaneous overexpression of *mnp4* and *aas*.

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