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Short communication

Isolation and heterologous expression of the *Phanerochaete chrysosporium* calmodulin gene

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ABSTRACT

We cloned complementary DNA (cDNA) corresponding to protein_id 10767 using transcripts extracted from a culture expressing ligninolytic enzymes, which is registered as a Ca^{2+}-binding protein in the *Phanerochaete chrysosporium* whole genome database. We used our results to correct an amino acid sequence present in the database. Phylogenetic and southern blot analyses of this re-predicted sequence suggest that this is the sole gene encoding calmodulin (*CaM*) in this fungus. A *P. chrysosporium* CaM protein (PcCaM) fused to a His-tag was expressed in *Escherichia coli*. The His-PcCaM showed appropriate CaM features.

White-rot fungi have efficient ligninolytic systems that completely decompose wood lignin into carbon dioxide and water (Kirk et al. 1975; Kirk and Farrell 1987). They also degrade persistent organic pollutants such as chlorinated dioxin (Bumpus et al. 1985) and azoic dyes (Cripps et al. 1990; Paszczynski et al. 1992; Spadaro et al. 1992). This ligninolytic ability can be useful for constructing a novel bioreactor system for the pretreatment of woody biomass prior to its conversion to various materials or for bioremediation. The ligninolytic ability of these fungi is attributable to many genes that are both known and unknown, and the inducing conditions and regulating factors are not completely understood. This lack of knowledge impedes the development of a highly effective lignin-degrading fungal strain for the construction of an efficient bioreactor system (Cullen and Kersten 2004).

Lignin peroxidase (LiP) and manganese peroxidase (MnP) are typical ligninolytic peroxidases that are thought to play an important role in the degradation of lignin (Cullen and Kersten 2004; Sugiura et al. 2012), chlorinated dioxin (Valli et al. 1992), and azoic dyes (Sedighi et al. 2009; Salame et al. 2010; Singh et al. 2010). The activities of these enzymes can be easily measured using culture filtrates. Hence, we have previously performed studies to search the master regulator gene by using ligninolytic enzyme genes as indicators (Minami et al. 2007, 2009; Sakamoto et al. 2010, 2012).

Phanerochaete chrysosporium is one of the most widely researched white-rot fungi globally, and a whole genome database of strain RP78 has been launched by the DOE Joint Genome Institute (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). The database predicts that the strain has 10

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isozyme genes encoding LiP and 5 genes encoding MnP (Martinez et al. 2004). Our previous studies indicated that a gene (protein_id 10767) homologous to calmodulin (CaM) is expressed in parallel with LiP and MnP expression in *P. chrysosporium* (Minami et al. 2007, 2009). In addition, the typical CaM antagonist N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) repressed the activity and the transcription of all isozyme genes (Sakamoto et al. 2010). However, it remained uncertain whether the homologous gene was truly a CaM-encoding gene, and its exact exon–intron boundaries and copy number were not confirmed.

CaM is a major Ca^{2+}-mediated signal transduction factor that contains 4 EF-hand motifs that bind Ca^{2+}. It regulates many physiological phenomena in eukaryotes via binding to at least 30 different targets/binding proteins, such as myosin light chain kinase, CaM-dependent kinases, and the phosphatase calcineurin (Means and Dedman 1980; Cheung 1980; Babu et al. 1985; Zhang and Lu 2003). CaMs are reported to be involved in hyphal growth, morphogenesis, germination, and penetration into plant tissues (Davis et al. 1986; Ahn et al. 2003; Sato et al. 2004; Wang et al. 2006; Ahn and Suh 2007; Kobayashi et al. 2007). Additionally, white-rot fungi may have a CaM-interacting protein that exclusively regulates the ligninolytic system downstream of CaM signaling, which would be a useful target for facilitating the development of a novel high lignin-degrading strain. Such proteins can be identified using protein–protein interaction analyses such as phage-display. However, before undertaking such analyses, the determination of the exact *CaM* gene is required.

1. Identification of the gene encoding CaM in *Phanerochaete chrysosporium*

We used the amino acid sequence of *Homo sapiens* CaM1 to perform a BLASTp search of the *P. chrysosporium* RP78 strain genome database for predicted proteins with homology to CaM. This search identified 6 amino acid sequences of putative Ca^{2+}-binding proteins (protein_ids: 6156, 9972, 10767, 10933, 135584, and 139216). These 6 amino acid sequences, as well as CaMs from other species, were compared by constructing a phylogenetic tree. However, the CaMs did not form a single clade. Interpretation of the tree was ambiguous and no *P. chrysosporium* CaM could be identified (Fig. S1; supplementary data). However, it was noted that the amino acid sequence of protein_id 10767 was the most homologous to CaMs from other species. We considered the possibility that misprediction of the exon regions of the gene corresponding to protein_id 10767 may cause misidentification of the amino acid sequence and could underlie this phenomenon. Therefore, we cloned and sequenced the full-length cDNA using 5′- and 3′-rapid amplification of cDNA ends (RACE). Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) from the *P. chrysosporium* strain RP78 mycelia during induction of MnP and LiP expression (Sakamoto et al. 2012). After treatment with RNase-free DNase (TaKaRa, Shiga, Japan), the extract was used to produce first-strand cDNA libraries for 3′-RACE and 5′-RACE, respectively, by using the SMARTer™ RACE cDNA Amplification Kit (TaKaRa). The second-strand cDNA libraries for the 5′- and 3′-untranslated regions were constructed via polymerase chain reaction (PCR) using the first-strand libraries as the template, along with specific primers for the gene corresponding to protein_id 10767 as follows: 5′-CGTCAGGAACTCGGGGAAA-3′ and 5′-CGGCACGTCATGAC CAA-3′, and PrimeSTAR® HS DNA Polymerase (TaKaRa). The PCR conditions used were as follows: 30 cycles, each consisting of denaturation at 98 °C for 10 s, annealing at 55 °C for 5 s, and elongation at 72 °C for 60 s. The resultant PCR products were over-hanged with adenine nucleotides on the 3′-ends by using Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania) at 72 °C for 60 s. The fragment was cloned into the pCR™ 2.1-TOPO® vector using the TOPO® TA Cloning® Kit (Invitrogen, California, USA), and it was sequenced. We found that the coding sequence predicted from the whole genome database lacks sequences from the section designated exon 2 as well as it lacks the complete sequence of the designated exon 3, as revealed by our cDNA analyses. Although we repeated cloning and sequencing 4 times, we did not find a splice variant of the gene. Thereafter, we used the newly predicted amino acid sequence of protein_id 10767 (accession no. BAM24398) to reconstruct the CaM phylogenic tree (Fig. 1). In this tree, the amino acid sequence and those from CaMs of other species formed a single clade, suggesting that the gene denoted by protein_id 10767 encodes *P. chrysosporium* CaM (PcCaM).

The re-predicted amino acid sequence of PcCaM has 4 EF-hand motifs binding calcium ions (Fig. 2) and is nearly identical to CaMs of other species (Fig. S2). This result shows that

Fig. 1 – Phylogenetic tree showing the relationship among amino acid sequences of CaMs of other species, including *Arabidopsis thaliana* CaM6 (accession no. NP_850860), *Aspergillus nidulans* (P60204), *Homo sapiens* CaM1 (NP_008819), *Magnaporthe grisea* (ACP19820), *Schizophyllum commune* (XP_003025993), and *Zea mays* (NP_001148310) and the candidate *Phanerochaete chrysosporium* CaMs, protein id 6156, 9972, 10933, 135584, 139216, and PcCaM. PcCaM is a re-predicted amino acid sequence of protein id 10767, which was determined by our cDNA sequence analyses (accession no. BAM24398). The phylogenetic tree was constructed using the neighbor-joining algorithm and 1000 bootstrapping replicates.

PcCaM is a highly conserved protein, as it is in other eukaryotic organisms. Two cAMP response element-binding (CREB) motifs were found in the promoter region using TFBIND (http://tfbind.hgc.jp/), which was released by Dr. Tsunoda to identify *cis*-elements that may be involved in transcription (Tsunoda and Takagi 1999). This is consistent with our previous study that indicated CaM transcription is regulated by cAMP signaling (Sakamoto et al. 2012). MacDonald et al. (1984) reported that intracellular cAMP levels increased during *P. chrysosporium* degradation of straw lignin. Boominathan and

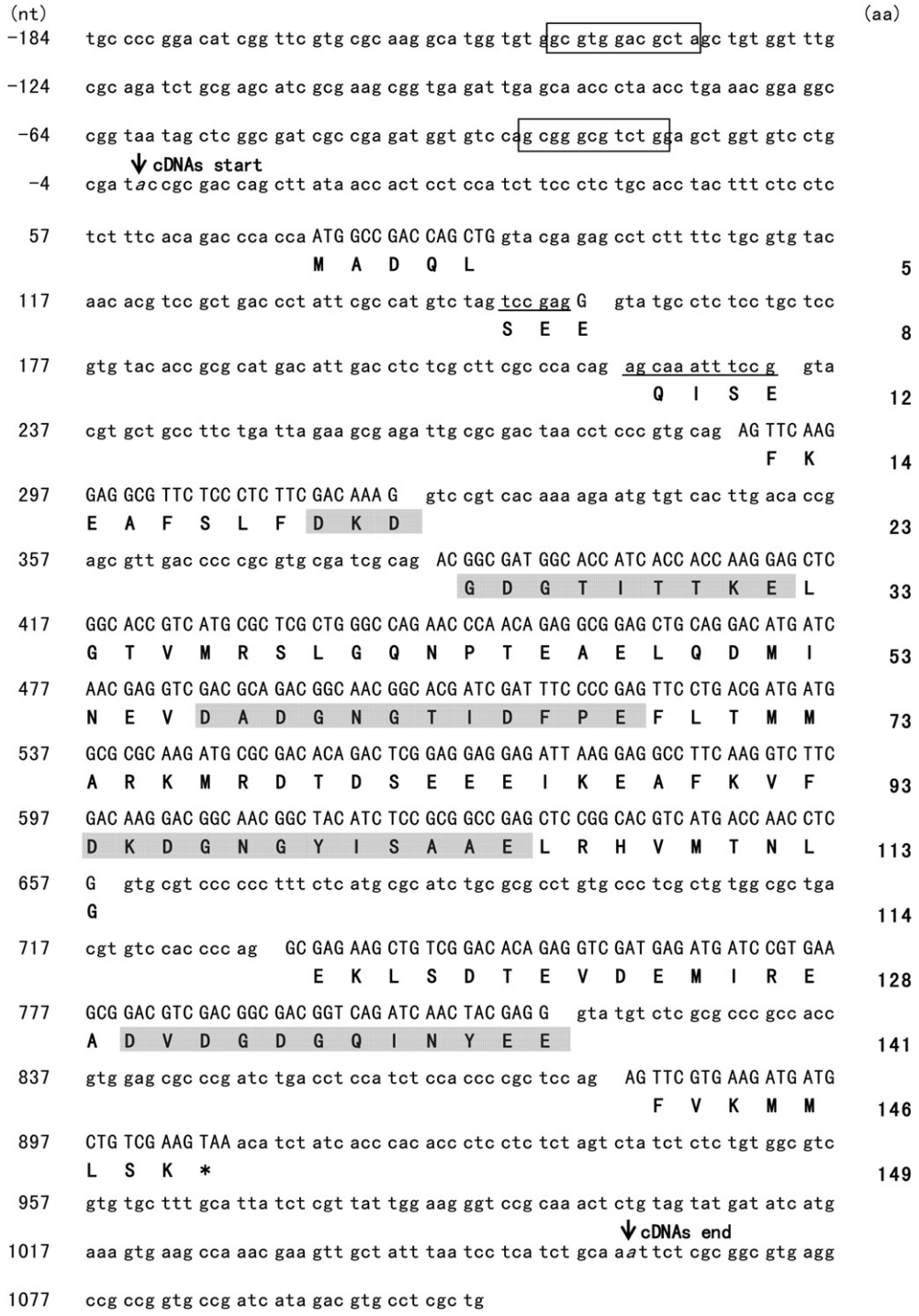

Fig. 2 – Nucleotide sequence and deduced amino acid sequence corresponding to protein_id 10767 in *Phanerochaete chrysosporium* RP78 (accession no. AB729130). Uppercase characters indicate the translated regions predicted from sequences within the whole genome database, and lowercase characters indicate the untranslated regions. The underlined regions, which were not contained in exons predicted by the whole genome database, were coding sequences newly revealed by this study. The amino acid sequence deduced in this study contains 149 amino acids. The shaded amino acid regions indicate the Ca^{2+}-binding domains. The 2 CREB motifs are boxed.

Reddy (1992) indicated that atropine application to *P. chrysosporium* cultures repressed LiP and MnP activity and decreased intracellular cAMP levels.

2. Southern blot analyses of the *PcCaM* gene

Southern blotting analyses were performed to estimate the copy number of the *PcCaM* gene in strain RP78. These analyses assess the probability that other *PcCaM* gene homologs exist in the strain using the AlkPhos Direct Labeling and Detection System (GE Healthcare). A fragment of the gene corresponding to protein_id 10767 was amplified with the following primer pair: 5′-GCCGCATATGGCCGACCAGCTGTCC-3′ and 5′-GCCGCTCGAGT TACTTCGACAGCATCA-3′ as the probe. Southern blots of *Bgl*II-, *Eco*RV-, or *Hin*dIII-digested genomic DNA were probed with the *PcCaM* DNA fragment. Only a single band signal was detected, irrespective of the restriction enzyme used, and the band sizes were consistent with those of nucleotide fragments from the region flanking the gene denoted by protein_id 10767, as predicted from the whole genome sequence (Fig. 3A and B). These results indicate that the homokaryotic strain RP78 harbors a single *CaM* gene.

3. Expression of PcCaM in *Escherichia coli*

The open reading frame (ORF) of the cloned cDNA corresponding to protein_id 10767, fused to a His-tag, was cloned into an expression vector, pCold TF DNA (TaKaRa). The resultant plasmid, pCold_PcCaM (Fig. S3; supplementary data), was introduced into One Shot® TOP10 chemically competent *E. coli* (Invitrogen). The transformed *E. coli* were grown at 37 °C to an OD_{600} of approximately 0.5 and were then transferred to 15 °C. After incubation for 30 min at 15 °C, isopropyl-β-D-1-thiogalactopyranoside was added at a final concentration of 1.0 mM, and the culture was incubated for 24 h at 15 °C with agitation at 130 rpm. The bacteria were harvested by centrifugation, and the His-PcCaM protein was purified according to the protocol of the QIAexpress® Ni-NTA Fast Start Kit (Qiagen, Dusseldorf, Germany).

The molecular weight of His-PcCaM was calculated to be 21 kDa [composed of a His-tag (4.0 kDa) and PcCaM (17 kDa)]. However, it is generally accepted that CaM migration during SDS-PAGE varies according to conformational changes in the molecule, which depends on Ca^{2+} ion binding (Jurado et al. 1999). Therefore, SDS-PAGE of CaM in the presence or absence of Ca^{2+} is often used as an indicator of the functionality of purified CaM protein. Fig. 4 shows faster mobility of His-PcCaM in the presence of Ca^{2+} compared to when Ca^{2+} was chelated by EGTA. This confirms that PcCaM is a CaM and indicates that the protein functionality was retained in the tagged protein.

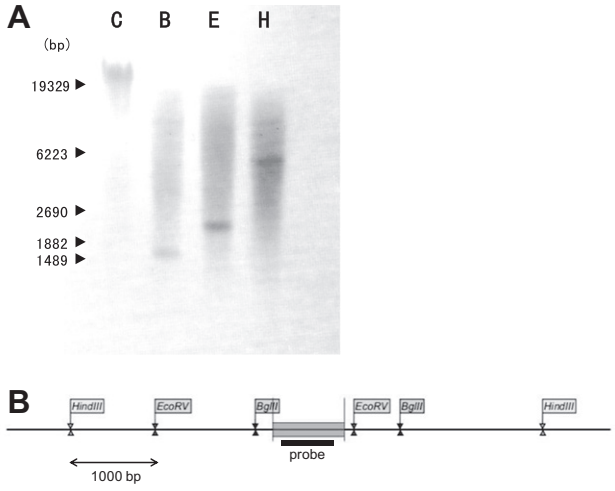

Fig. 3 – Copy number estimation of the *Phanerochaete chrysosporium CaM* gene in strain RP78. (A) Southern blot analyses of *P. chrysosporium* strain RP78 genomic DNA digested with *Bgl*II (lane B), *Eco*RV (lane E), and *Hin*dIII (lane H), and undigested DNA (lane C) and probed with a PCR fragment amplified from the gene corresponding to protein_id 10767. (B) Restriction map of the region containing the gene corresponding to protein_id 10767.

4. Interaction between PcCaM and PcCaNA

We confirmed the ability of His-PcCaM to bind CaM-interacting proteins by performing an interaction assay in which His-PcCaM was used to pull down GST-*P. chrysosporium* calcineurin (PcCaNA) expressed in *E. coli*. CaNA is a typical CaM-interacting protein. Western blotting analyses were performed using the GST antibody, and the findings showed that His-PcCaM bound to GST-PcCaNA, whereas GST alone did not interact with His-PcCaM (Fig. 5). These results again confirm that His-PcCaM is a functionally active protein.

His-PcCaM is useful for protein–protein interaction analyses, such as phage-display, in order to identify CaM-interacting proteins that exclusively regulate the ligninolytic system of fungi downstream of CaM signaling. These interacting proteins may be potential candidates as the important regulatory genes for lignin-degrading capacity.

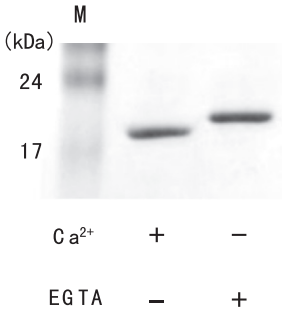

Fig. 4 – Ca^{2+}-dependent mobility shift of His-PcCaM during SDS-PAGE. Purified His-PcCaM was incubated for 30 min at room temperature in Ca^{2+}buffer [TBST buffer: 25 mM Tris–HCl (pH 7.4), 137 mM NaCl, 2.68 mM KCl, 0.1% Tween-20, including 2 mM $CaCl_2$] or in ethylene glycol tetraacetic acid (EGTA) buffer (TBST buffer including 5 mM of EGTA, a Ca^{2+} chelator). Subsequently, the proteins were electrophoresed on a 15% SDS-PAGE gel (e-PAGEL; Atto, Tokyo, Japan), and they were stained with Coomassie Brilliant Blue R250.

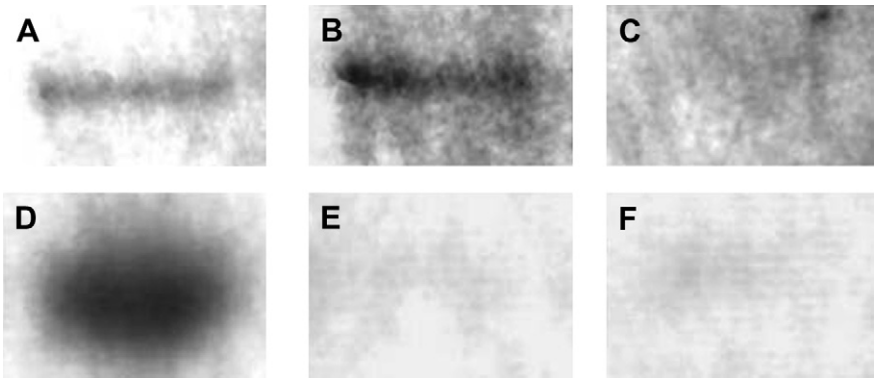

Fig. 5 – Pull-down analyses of His-PcCaM using western blot detected by the GST antibody. The ORF sequence of *PcCaNA* corresponding to protein_id 131295 was amplified from first-strand cDNA libraries by using the following specific primers (5′-CACCATGAGTGCCACACAAAAGCATA-3′ and 5′-TCTACTCGAAGGGGCGGCCGGG-3′) and PrimeSTAR® HS DNA Polymerase (TaKaRa). The PCR product was cloned into the pDEST™15 vector (Invitrogen) to produce a PcCaNA protein fused to a glutathione-S-transferase (GST) tag. This construct was introduced into *Escherichia coli* BL21-AI™ One Shot® chemically competent *E. coli* (Invitrogen), according to the supplier's instructions. The transformed *E. coli* cells were grown at 37 °C to an OD_{600} of approximately 0.4. L-arabinose was added at a final concentration of 0.2% to induce fusion gene expression, and the culture was incubated for 3 h at 37 °C. Bacteria were lysed using xTractor buffer (TaKaRa) for His pull-down assays. His-Accept agarose (Nacalai Tesque, Kyoto, Japan) coated with His-PcCaM was incubated for 1 h at 4 °C with the lysate in Ca^{2+} buffer, followed by 5 washes with Ca^{2+} buffer or EGTA buffer. The resultant complex of His-PcCaM and PcCaNA was eluted with imidazole buffer [250 mM imidazole, 50 mM Na-phosphate (pH 8.0), and 300 mM NaCl]. The PcCaNA was dissociated from the complex in SDS-PAGE, followed by western blotting using the GST antibody (GE Healthcare). (A), (B), and (C) using recombinant *E. coli* lysates expressing GST-PcCaNA for analyses; (D), (E), and (F) recombinant *E. coli* lysates expressing only GST. (A) and (D) control lysates; (B) and (E) proteins pulled-down from the His-tagged PcCaM lysate; (C) and (F) proteins pulled-down from the lysate without His-PcCaM (using only His-Accept agarose).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.myc.2012.09.017.

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