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SHORT COMMUNICATION

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Isolation, characterization, and expression analysis of a class IV chitin synthase gene from the edible basidiomycetous mushroom *Pleurotus ostreatus*

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Abstract We isolated and characterized the class IV chitin synthase gene (*Pochs1*) from the edible basidiomycetous mushroom, *Pleurotus ostreatus*. Real-time quantitative RT-PCR analysis of the transcriptional expression pattern of *Pochs1* in the course of fruit-body formation of *P. ostreatus* revealed that the transcriptional level of *Pochs1* is higher in the stage of immature fruit body than in the stages of mycelia and mature fruit body. Furthermore, the transcriptional level of *Pochs1* in mycelia was shown to have increased significantly by the temperature-downshift treatment, which is one of the important factors for inducing the onset of fruit-body formation of *P. ostreatus*.

Key words Basidiomycetous mushroom · Chitin synthase · Fruit body · Morphogenesis · *Pleurotus ostreatus*

The morphological differentiation from mycelia to fruit body in basidiomycetous mushrooms is an interesting and important biological process on both scientific and commercial aspects. Elucidation of the molecular mechanism of the process in fruit-body formation is expected to lead to more efficient procedures for mushroom production, utilization of fruit-body specific materials, and breeding to improve strain characters. However, the mechanism is still poorly understood and needs far more detailed investigation.

Cell wall integrity is thought to be very important for fungal morphogenesis. Chitin, a β-1, 4-linked homopolymer of *N*-acetylglucosamine units, is one of the major structural components of cell walls of a wide range of filamentous fungi, including basidiomycetes (Bartnicki-Garcia 1968; Bulawa 1993). Therefore, understanding of the mechanism of chitin biosynthesis in filamentous fungi may offer important insights into fungal morphogenesis (Bulawa 1993). Polysaccharide chitin biosynthesis is performed by chitin synthases (EC 2.4.1.16), which are membrane-bound proteins (Durán et al. 1975). Fungi produce multiple chitin synthase isozymes, which have been classified into at least six classes (class I–VI) based on their conserved region structures (Bowen et al. 1992; Roncero 2002; Ruiz-Herrera et al. 2002; Niño-Vega et al. 2004). Along these lines, the roles of chitin synthases in the determination of morphology and regulation mechanisms of their genes have been studied mainly with ascomycetes (Chigira et al. 2002; Ichinomiya et al. 2002; Müller et al. 2002; Roncero 2002; Takeshita et al. 2002, 2005, 2006; Wang et al. 2002); however, little information is available from basidiomycetous mushrooms. To study the mechanism of fruit-body formation of basidiomycetous mushrooms in terms of chitin synthesis, we attempted to analyze a chitin synthase gene from *Pleurotus ostreatus* (Jacq: Fv.) Kummer, the so-called oyster mushroom, which is one of the most widely cultivated edible basidiomycetous mushrooms. This article constitutes the first contribution to the study of the class IV chitin synthase gene in basidiomycetous mushrooms.

The strain used in this study was *P. ostreatus* AM-1 (dikaryon), which was a stock culture of our laboratory originated from a commercial source and maintained on potato dextrose agar (PDA) medium (Becton Dickinson, Baltimore, MD, USA) at 4°C with periodic subculture. The mycelia were grown on PDA medium at 25°C in the dark. To isolate a chitin synthase gene fragment, polymerase chain reaction (PCR) amplification was run with the *P. ostreatus* genomic DNA as a template and the degenerate primer set already designed to amplify the parts of chitin synthase genes by Specht et al. (1996). *Pleurotus ostreatus* genomic DNA was prepared by the cetyltrimethylammonium bromide (CTAB) method (Klimyuk et al. 1993) from the mycelia grown in a potato dextrose broth medium (Becton Dickinson). A 378-bp DNA fragment was amplified. The sequence analysis revealed that this DNA fragment encodes a polypeptide highly homologous to parts of other fungal chitin synthases [for example, 89% identity to the class IV chitin synthase gene of *Ustilago maydis* (DC.)

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Corda], indicating that this DNA fragment was presumed to be a part of the *P. ostreatus* chitin synthase gene (which we designated *Pochs1*). To isolate the unknown upstream and downstream regions of *Pochs1,* thermal asymmetrical interlaced (TAIL)-PCRs were carried out according to the protocol previously described by Liu and Whittier (1995). Then, we amplified the 5575-bp genomic region containing the whole coding sequence of *Pochs1* by PCR using *P. ostreatus* genomic DNA as a template and primers synthesized according to the sequences that became known by the TAIL-PCRs. We determined the 5575-bp sequence by direct sequencing of the uncloned PCR product mentioned above on both strands.

In the process of nucleotide sequence determination, we detected no ambiguities and errors arising from the existence of the allele with a different sequence. To confirm the translation initiation codon of *Pochs1* and to obtain the cDNA sequence, we determined the 5′-cDNA end by means of 5′-rapid amplification of cDNA end (5′-RACE). The 5′-RACE experiment was carried out using a SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA), following the manufacturer's instructions. To determine the complete sequence of *Pochs1* cDNA, the DNA fragments of cDNA were amplified by RT-PCR, using several specific primers designed on the basis of the *Pochs1* sequence already mentioned, and then sequenced. The total RNA was isolated from *P. ostreatus* mycelia using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase I (Takara Bio, Otsu, Shiga, Japan) to remove contaminating DNA. Reverse transcription was carried out using SuperScript III RNaseH$^-$ Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. We determined the sequence of *Pochs1* cDNA containing the whole coding region. The genomic DNA and cDNA sequences of *Pochs1* are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB263750 and AB262583, respectively. A comparison of genomic DNA and cDNA sequences revealed that the *Pochs1* gene has a coding capacity of 1436 amino acid residues and is interrupted by four small introns (49 to 62 bp) (Fig. 1). All intron–exon boundaries conformed to the GT-AG splicing rule (Lerner et al. 1980). The promoter region of *Pochs1* contains a CAAT box (at position –124 to –119 relative to the translation start codon), two GC box-like sequences (–265 to –260 and –220 to –215) and a CT-stretch (–90 to –65), but not a typical TATA box. The CT-stretch is located in the region immediately upstream of the putative transcription starting point (–64) predicted from the 5′-RACE experiment.

This result is consistent with the previous observation that the transcription starting points of various basidiomycetous fungal genes are often found in or near the CT-stretches (Yamazaki et al. 2004). Two polyadenylation signal-like sequences were found in the 3′-flanking region. The deduced amino acid sequence of the gene product (PoChs1) showed notable similarities to those of class IV chitin synthases of other fungi, with the highest degree of identity (51%) to that of class IV chitin synthase (UmChs5) from *U. maydis* (Xoconostle-Cázares et al. 1997). The phylogenetic tree constructed using the ClustalW program (http://align.genome.jp/clustalw/) also demonstrated that PoChs1 belongs to the class IV group of chitin synthases (data not shown). Sequence alignment analysis of PoChs1 and other fungal class IV chitin synthases indicated that the C-terminal-half region of PoChs1 is highly homologous to those of the members of this class and contains LGEDRYL and SQRRRW sequences, which are implicated as essential catalytical sites (Nagahashi et al. 1995; Yabe et al. 1998) (Fig. 2). Hydrophobicity analysis by the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/) suggested that PoChs1 contains four putative transmembrane domains.

Because the transcriptional regulation of chitin synthase genes is considered to be one of the important regulatory mechanisms for fungal chitin synthesis and therefore may be closely related to the fungal morphogenesis (Chen-Wu et al. 1992; Pammer et al. 1992; Sudoh et al. 1993; Choi et al. 1994; Motoyama et al. 1994; Xoconostle-Cázares et al. 1997; Munro et al. 1998; Wang and Szaniszlo 2000), we examined the transcriptional expression patterns of *Pochs1* in the course of fruit-body formation and in the different tissues of fruit bodies (stipes and pilei) of *P. ostreatus* by means of real-time quantitative reverse transcriptase (RT)-PCR. For the production of fruit bodies, the mycelia were inoculated on sawdust medium supplemented with rice bran (sawdust : rice bran = 1 : 1 w/w) in 200-ml bottles (45 g medium in a bottle). The cultures were incubated at 25°C for 2 weeks in the dark, and subsequently the fruit bodies were induced by the conventional fruiting treatment as follows: removing the mycelial layers of the top surfaces of the cultures by scratching, watering the cultures, and then transferring the cultures into the condition of continuous illumination at 15°C and 90% humidity. Total RNA samples were prepared as already described from three distinct developmental stages: mycelia (before fruiting treatment was conducted), immature fruit bodies, and mature fruit bodies. Each fruit body was separated into stipe and pileus before RNA extraction. The RNA samples were subjected to first-strand cDNA synthesis using SuperScript III RNaseH$^-$ Reverse Transcriptase (Invitrogen). Real-time quantitative RT-PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), according to the manufacturer's instructions, on an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The *Pochs1*-specific primer set, 5′-TACCTCCTCTCCTT GCCCATC-3′ and 5′-TCTTTGTCCTCTCCAGCAACC-3′, generating the sole amplicon (110 bp), was used. The levels of 18S rRNA were used as an internal control using the following primer set: 5′-TACACTGACAGAGCCA GCGAG-3′ and 5′-GGACGTAATCAACGCGAGCTG-3′, generating a 154-bp amplicon. As shown in Fig. 3A, *Pochs1* was transcribed at all developmental stages examined in this study (mycelia before fruiting treatment, and immature and mature fruit bodies); however, the transcriptional level was different in the three stages and higher in the immature fruit body (both stipe and pileus) than in mycelia and mature fruit body. This observation suggests that the *Pochs1* gene is transcribed more actively at the early stage of fruit-body formation after fruiting treatment

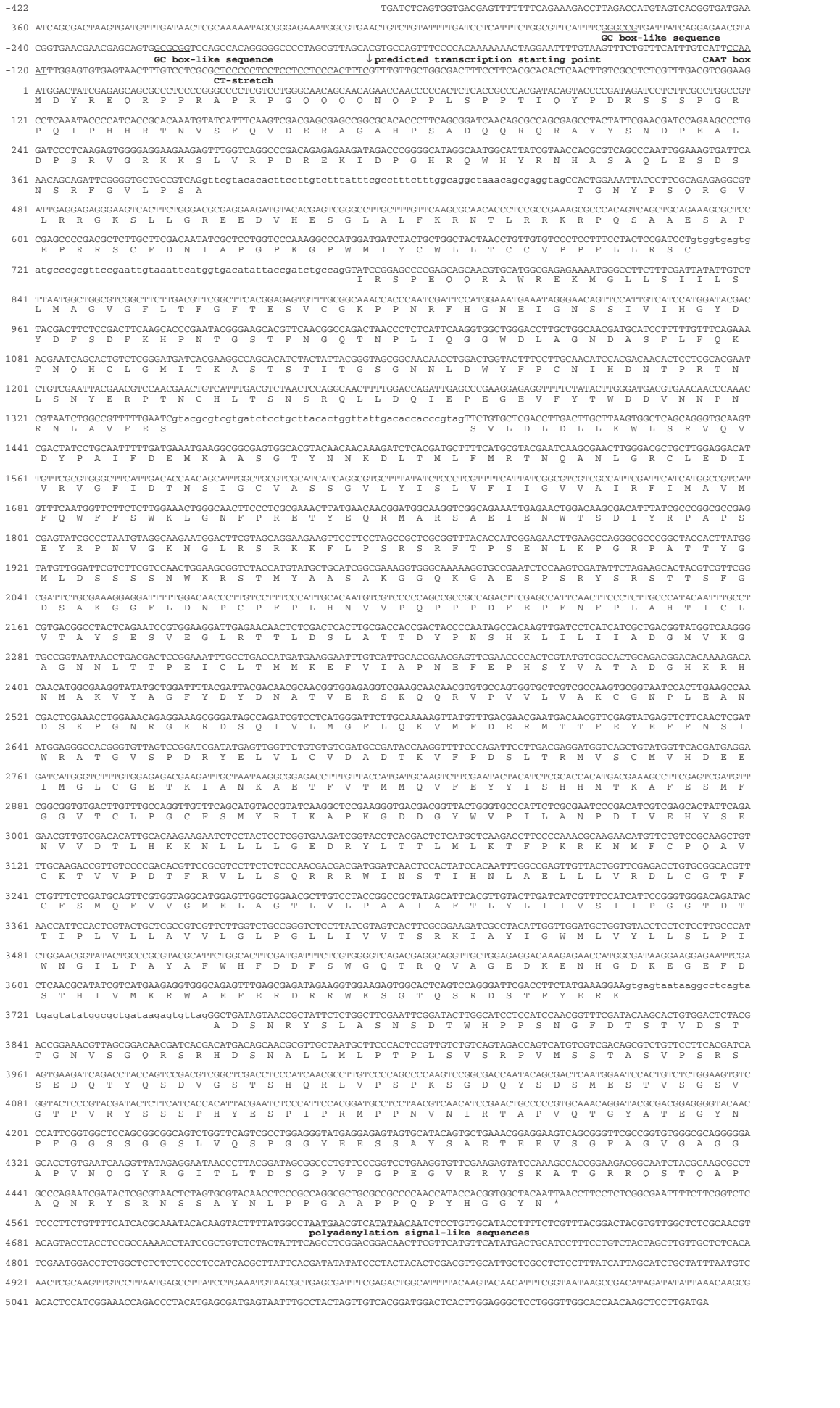

Fig. 1. Nucleotide and deduced amino acid sequences of *Pochs1*. Introns are indicated by *lowercase letters*. *Arrow* indicates the predicted transcription starting point. The CAAT box, CT-stretch, two GC box-like sequences, and two polyadenylation signal-like sequences are *underlined*

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PoChs1    PPPDFEPFNFPLAHTICLVTAYSESVEGLRTTLDSLATTDYPNSHKLILIIADGMVKGAGNNLTTPEICLTMMKEFVIAPNEFEPHSYVATADGHKRHNM  746
UmChs5    PGPDYRPFGFQLAHSICLVTAYSESFEGLRTTLDSLATTDYPNSHKLLLVIADGIVKGAGSDISTPDICLSMMKDLVIPAEEVEGNSYVAIADGYKRHNM  762
NcChs4    PPSDWMPFGFPLAHTICLVTAYSEGEMGVRTTLDSIAMTDYPNSHKVILVICDGIIKGHGEEHSTPDIILGMMKDHTIHPDDVEPFSYVAVATGSKRHNM  734
GvChs3    PPPEYQPFGFPLAHTMCLITCYSEGEQGVRSTLDSIATTDYPNSHKLMLIVCDGLVKGSGETLTTPEIILSMMKDHAVHPDDITPFSYVAVASGSKRHNM  777
AnChsD    PPPDWQPYGFPLAHAMCLVTCYSEGEEGIRTTLDSIALTDYPNSHKSIVVICDGIIKGKGEEFSTPDIVLRMMRDPIIPPEEVEAFSYVAVATGSKRHNM  754
BcChsIV   PPKEWEPYGFPLAHAILLVTAYSEGELGIRTTLDSIATTDYPNSHKTILVICDGIIKGEGEPKATPEIVLGMMKDFVTPVDEVPAYSYVAVERGSKRHNM  771
CaCHS3    PPVEYQPFGYPLAHTINLVTCYSEDEEGIRITLDSIATTDYPNSHKLILVICDGIIKGSGNDETTPDIVLDMMSDLTVPRDEVEAYSYVAVAQGSKRHNM  749
ScCHS3    PPLDFMPYGFPLIHTICFVTCYSEDEEGLRTTLDSLSTTDYPNSHKLLMVVCDGLIKGSGNDKTTPEIALGMMDDFVTPPDEVKPYSYVAVASGSKRHNM  739
          *    *   * *    * ***  * * ****  ********     **  ** *   ** * * **           ****   * *****

PoChs1    AKVYAGFYDYDNATVER-SKQQRVPVVLVAKCGNPLEANDSKPGNRGKRDSQIVLMGFLQKVMFDERMTTFEYEFFNSIWRATGVSPDRYELVLCVDADT  845
UmChs5    CKIYAGFYDYDDETVER-SKQQRVPMILVAKCGTPLEADSAKPGNRGKRDSQVLLMAFMQKVMFDERMTAFEYEFFNSIWRVTGVSPDNYEIVLCVDADT  861
NcChs4    AKVYTGFYDYGTNSAIPLEKQQRVPMMMVVKCGTPAEASKSKPGNRGKRDSQIILMSFLQKVMFDERMTELEYEMFNGLWKITGISPDFYEIVLMVDADT  834
GvChs3    AQVYAGFYDYGEQSMVPVEKQQRVPMLVVVKCGTPAEKSAKKPGNRGKRDSQIILMSFLQKVMFDERMTELEYEMFNGIWKVTGISPDYYETVLMVDADT  877
AnChsD    AKVYAGFYDYGEHSIIPVEKQQRVPMMIIVKCGTPAEATAAKPGNRGKRDSQIILMSFLQKVMFDERMTELEYEMFNGLLHVTGIPPDFYEVVLMVDADT  854
BcChsIV   AKVYAGFYDYGADSTINVNRQLRVPMVCIVKCGTPDEATHRKPGNRGKRDSQIILMSFLQKVMFDERMTELEFEIFNGFYQISGIFPDMYEVVLMVDADT  871
CaCHS3    AKVYAGFYKYN-DETVPPEKQQRIPMITIVKCGTPEEASAPKPGNRGKRDSQIILMSFLQKVVFDERMTSLEYEMLQSIWRITGLMAEFYEIVLMVDADT  848
ScCHS3    AKIYAGFYKYD-DSTIPPENQQRVPIITIVKCGTPAEQGAAKPGNRGKRDSQIILMSFLEKITFDERMTQLEFQLLKNIWQITGLMADFYETVLMVDADT  838
             * *** *          * * *     *** * *    ***********  ** *  *  ******  *          *     ** ** *****

PoChs1    KVFPDSLTRMVSCMVHDEEIMGLCGETKIANKAETFVTMMQVFEYYISHHMTKAFESMFGGVTCLPGCFSMYRIKAPKGDDGYWVPILANPDIVEHYSEN  945
UmChs5    KVFPDSLSRMVACMVEDPEIMGLCGETKIANKSETWVTMIQVFEYYISHHQTKAFEACFGGVTCLPGCFSAYRIKAPKGPHGYWVPILANPDIVEHYSEN  961
NcChs4    KVFPDSLTHMISAMVKDPEIMGLCGETKIANKRASWVSAIQVFEYFVSHHLAKAFESVFGGVTCLPGCFCMYRIKAPKGAQNYWVPILANPDVVEHYSEN  934
GvChs3    KVFPDSLTHMVGAMVHDPEIMGLCGETKIANKRTSFVSMMQVFEYFISHHQTKAFESVFGGVTCLPGCFCMYRIKAPKGGQNYWVPILANPDVVEHYSED  977
AnChsD    KVFPDSLTHMISAMVKDPEVMGLCGETKIANKTDSWVTMIQVFEYFVSHHQSKAFESVFGGVTCLPGCFSMYRIKAPKGGQNYWVPILANPDIVEHYSEN  954
BcChsIV   KVFPDSLTHMVSAMVKDPEIMGLCGETKIANKRASFTTAIQVFEYFISHHSVKSFESIFGGVTCLPGCFSMYRIKAPKGGN-YWVPILANPDVVEHYSDN  970
CaCHS3    KVFPDSLTHMVAEMVKDPTIMGLCGETKISNKAQTWVTAIQVFEYYISHHQAKAFESIFGGVTCLPGCFCMYRIKAPKGSDGYWVPILANPDIVERYSDN  948
ScCHS3    KVFPDALTHMVAEMVKDPLIMGLCGETKIANKAQSWVTAIQVFEYYISHHQAKAFESVFGSVTCLPGCFSMYRIKSPKGSDGYWVPVLANPDIVERYSDN  938
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PoChs1    VVDTLHKKNLLLLGEDRYLTTLMLKTFPKRKNMFCPQAVCKTVVPDTFRVLLSQRRRWINSTIHNLAELLLVRDLCGTFCFSMQFVVGMELAGTLVLPAA 1045
UmChs5    VVDTLHKKNLLLLGEDRYLTTLMLKTFPKRKMMFVPQAVCKTIVPDTFRILLSQRRRWINSTVHNLFELVMVNDLCGTFCFSMRFVVFMELTGTLVLPAA 1061
NcChs4    VVDTLHKKNLLLLGEDRYLSTLMLRTFPKRKQVFVPQAVCKTTVPDEFMVLLSQRRRWINSTIHNLMELVLVRDLCGTFCFSMQFIVGIELIGTLVLPAA 1034
GvChs3    VVDTLHTKNLLLLGEDRYLSTLMLKTFPKRKQIFVPQAVCKTIVPDSFGVLLSQRRRWINSTVHNLMELVLVRDLCGTFCFSMQFVVFIELIGTVVLPAA 1077
AnChsD    VVDTLHKKNLLLLGEDRYLSTLMLRTFPKRKQIFVPQAVCKTVVPDKFMVLLSQRRRWINSTVHNLMELVLVRDLCGTFCFSMQFVIFVELVGTVVLPAA 1054
BcChsIV   VVDTLHKKNLLLLGEDRYLSTLMLRTFPKRKQVFVPQAVCKTQVPDSLWILMSQRRRWINSTVHNLMELVLVRDLCGTFCFSMQFVVFIDLVSTLVLPAA 1070
CaCHS3    VVDTLHRKNLLLLGEDRYLSSLMLRTFPTRKQVFVPKAACKTVVPDKFKVLLSQRRRWINSTVHNLFELVLVKDLCGTFCFSMQFVIFIELIGTLVLPAA 1048
ScCHS3    VTNTLHKKNLLLLGEDRFLSSLMLKTFPKRKQVFVPKAACKTIAPDKFKVLLSQRRRWINSTVHNLFELVLIRDLCGTFCFSMQFVIGIELIGTMVLPLA 1038
          *  *** ********** *  *** *** **  * * * ***  **    * ********** *** **    ********** *     *  * *** *
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Fig. 2. Comparison of the deduced amino acid sequences of PoChs1 and other fungal class IV chitin synthases. Multiple sequence alignment was made using the ClustalW program at GenomeNet (http://align.genome.jp/clustalw/). Only regions displaying high homology (C-terminal half regions) are shown. Identical residues among all aligned sequences are shown by *asterisks*. Two highly conserved domains, which are implied as essential catalytical sites of chitin synthases, are *overlined*. *UmChs5*, *Ustilago maydis* UmChs5 (accession number O13394); *NcChs4*, *Neurospora crassa* Shear & B.O. Dodge Chs4 (Q01285); *GvChs3*, *Glomus versiforme* (Karsten) Berch GvChs3 (AJ009630); *AnChsD*, *Aspergillus nidulans* (Eidam) Vuill ChsD (P78611); *BcChsIV*, *Botryotinia fuckeliana* (de Bary) Whetzel ChsIV (AAF19527); *CaCHS3*, *Candida albicans* (C.P. Robin) Berkhout CHS3 (P30573); *ScCHS3*, *Saccharomyces cerevisiae* Meyen ex E.C. Hansen CHS3 (P29465)

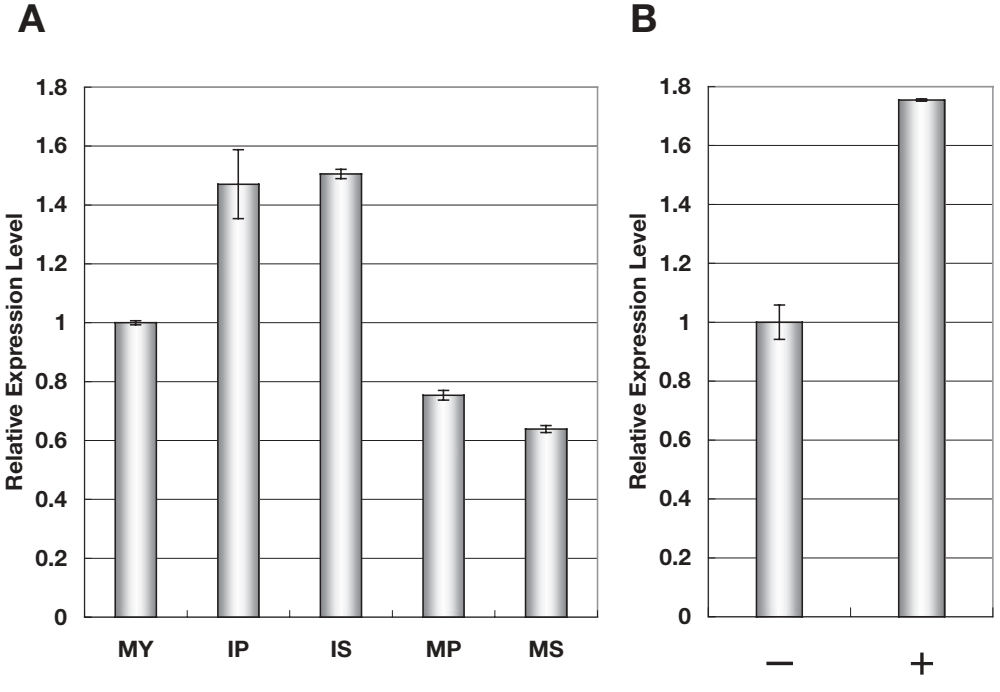

Fig. 3. Real-time quantitative reverse transcriptase-polymerase reaction (RT-PCR) analyses of *Pochs1* expression. Data represent the means and error bars for three independent experiments. **A** Relative expression levels during the fruit-body formation of *Pleurotus ostreatus*. Experimental samples were prepared from mycelia (*MY*), pileus of immature fruit body (*IP*), stipe of immature fruit body (*IS*), pileus of mature fruit body (*MP*), and stipe of mature fruit body (*MS*). **B** Comparison of relative expression levels of *Pochs1* between mycelia with (+) and without (–) the temperature-downshift treatment

is conducted and therefore might play an important role at the same stage.

This result led us to examine the effect of the temperature-downshift treatment, which is one of the important factors for inducing the onset of fruit-body formation (fruiting treatment) of *P. ostreatus*, on the transcriptional level of *Pochs1* in mycelia. The mycelia were grown on the sawdust-based medium already described at 25°C in the dark for 10 days, and subsequently the culture was continued for an additional 5 days, either at 15°C in the dark (the temperature-downshift treatment: +) or at 25°C in the dark (the temperature-downshift treatment: –). The analysis of the transcriptional levels of *Pochs1* in both mycelia (+ and –) was carried out as already described. As shown in Fig. 3B, the transcriptional level in mycelia increased significantly (about 1.8 fold) in response to the temperature-downshift treatment. Based on this result, we suggest that the *Pochs1* gene is upregulated by at least one of the fruiting treatment factors and may be correlated with the process in the fruit-body formation of *P. ostreatus*.

To examine the effects of other factors for inducing the fruit-body formation, such as light, moisture conditions, and nutritional conditions, on *Pochs1* expression seems to be interesting and is now underway. An increase (1.9 fold) in the expression level of a gene encoding Δ9 fatty acid desaturase in mycelia by the temperature-downshift treatment was also observed in the edible basidiomycetous mushroom *Lentinula edodes* (Berk.) Pegler (Sakai and Kajiwara 2003), and the authors suggested the correlation of the gene with fruit-body formation. In contrast to our results as reported here, Sreenivasaprasad et al. (2000) reported that the transcriptional level of the class III chitin synthase gene of *Agaricus bisporus* (J. E. Lange) Pilát was very low at the mycelial stage and high in the fruit bodies, and that no detectable difference in the transcriptional level was observed among fruit bodies at the different maturation stages. The different behaviors observed between class III and class IV genes, although their origins are different, suggested that chitin synthases belonging to different classes may have different functions in the fruit-body formation of the basidiomycetous mushrooms. The chitin synthase genes of other classes from *P. ostreatus* remain to be studied for the better understanding of the correlation between chitin synthesis and fruit-body formation in *P. ostreatus*.

In the present study, we have reported the isolation and characterization of a class IV chitin synthase gene, *Pochs1*, from the edible basidiomycetous mushroom *P. ostreatus.* Furthermore, we showed that *Pochs1* is temporally regulated at the transcriptional level during fruit-body formation and that the transcriptional level is increased in response to temperature-downshift treatment.

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