Mikihiro Nishihara • Akira Watanabe • Yasuhiko Asada

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

Received: November 7, 2006 / Accepted: December 27, 2006


#### Abstract

We isolated and characterized the class IV chitin synthase gene (Pochs1) from the edible basidiomycetous mushroom, Pleurotus ostreatus. Real-time quantitative RTPCR 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 $\beta-1,4$-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 impor-

[^0]tant 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 $4^{\circ} \mathrm{C}$ with periodic subculture. The mycelia were grown on PDA medium at $25^{\circ} \mathrm{C}$ 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.)

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 Pochs 1 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 $5^{\prime}$-cDNA end by means of $5^{\prime}$-rapid amplification of cDNA end ( $5^{\prime}$-RACE). The $5^{\prime}$ 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 Pochsl 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 Pochsl 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 Pochsl 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 GTAG 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 CTstretches (Yamazaki et al. 2004). Two polyadenylation signal-like sequences were found in the $3^{\prime}$-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 $=1: 1 \mathrm{w} / \mathrm{w})$ in $200-\mathrm{ml}$ bottles $(45 \mathrm{~g}$ medium in a bottle). The cultures were incubated at $25^{\circ} \mathrm{C}$ 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 $15^{\circ} \mathrm{C}$ 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, $5^{\prime}$-TACCTCCTCTCCTT GCCCATC-3' and $5^{\prime}$-TCTTTGTCCTCTCCAGCAACC$3^{\prime}$, generating the sole amplicon ( 110 bp ), was used. The levels of 18 S rRNA were used as an internal control using the following primer set: 5'-TACACTGACAGAGCCA GCGAG- $3^{\prime}$ and $5^{\prime}$-GGACGTAATCAACGCGAGCTG$3^{\prime}$, 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 boxlike sequences, and two polyadenylation signal-like sequences are underlined
$-422$
TGATCTCAGTGGTGACGAGTTTTTTTCAGAAAGACCTTAGACCATGTAGTCACGGTGATGAA
-360 ATCAGCGACTAAGTGATGTTTGATAACTCGCAAAAATAGCGGGAGAAATGGCGTGAACTGTCTGTATTTTGATCCTCATTTCTGGCGTTCATTTCGGGCCGTGATTATCAGGAGAACGTA
-240 CGGTGAACGAACGAGCAGTGGCGCGGTCCAGCCACAGGGGGCCCCTAGCGTTAGCACGTGCCAGTTTCCCCACAAAAAAACTAGGAATTTTGTAAGTTTCTGTTTCATTTGTCATTCCAA -120 AC box-like sequencer ATTGGAGTGTGAGTAACTTTGTCCTCGCGCTCCCCCTCCTCCTCCTCCCACTTTCGTTTGTTGCTGGCGACTTTCCTTCACGCACACTCAACTTGTCGCCTCTCGTTTGACGTCGGAAG 1 ATGGACTATCGAGAGCAGCGCCCTCCCCGGGCCCCTCGTCCTGGGCAACAGCAACAGAACCAACCCCCACTCTCACCGCCCACGATACAGTACCCCGATAGATCCTCTTCGCCTGGCCGT 21 CСTCAAATACCCCATCACCGCACAAATGTATCATTTCAAGTCGACGAGCGAGCCGGCGCACACCCTTCAGCGGATCAACAGCGCCAGCGAGCCTACTATTCGAACGATCCAGAAGCCCTG

241 GATCCCTCAAGAGTGGGGAGGAAGAAGAGTTTGGTCAGGCCCGACAGAGAGAAGATAGACCCGGGGCATAGGCAATGGCATTATCGTAACCACGCGTCAGCCCAATTGGAAAGTGATTCA


481 ATTGAGGAGAGGGAAGTCACTTCTGGGACGCGAGGAAGATGTACACGAGTCGGGCCTTGCTTTGTTCAAGCGCAACACCCTCCGCCGAAAGCGCCCACAGTCAGCTGCAGAAAGCGCTCC

601 CGAGCCCCGACGCTCTTGCTTCGACAATATCGCTCCTGGTCCCAAAGGCCCATGGATGATCTACTGCTGGCTACTAACCTGTTGTGTCCCTCCTTCCTACTCCGATCCTgtgetgagt

721 atgcccgcgttccgaattgtaaattcatggtgacatattaccgatctgccagGTATCCGGAGCCCCGAGCAGCAACGTGCATGGCGAGAGAAAATGGGCCTTCTTTCGATTTATATTGTCT $\begin{array}{lllllllllllllllll}R & S & E & Q & R & A & R & E & K & M & L & L & S & I & L & S\end{array}$
 61 TACGACTTCTCCGACTTCAAGCACCCGAATACGGGAAGCACGTTCAACGGCCAGACTAACCCTCTCATTCAAGGTGGCTGGGACCTTGCTGGCAACGATGCATCCTTTTTGTTTCAGAAA


1081 ACGAATCAGCACTGTCTCGGGATGATCACGAAGGCCAGCACATCTACTATTACGGGTAGCGGCAACAACCTGGACTGGTACTTTCCTTGCAACATCCACGACAACACTCCTCGCACGAAT 1201 CTGTCGAATTACGAACGTCCAACGAACTGTCATTTGACGTCTAACTCCAGGCAACTTTTGGACCAGATTGAGCCCGAAGGAGAGGTTTTCTATACTTGGGATGACGTGAACAACCCAAAC

 1441 CGACTATCCTGCAATTTTTGGATGAAATGAAGGCGGCGAGTGGCACGTACAACAACAAAGATCTCACGATGCTTTTTCATGCGTACGAATCAAGCGAACTTGGGACGCTGCTTGGAGGACAT D P A F D E M K A S G T Y N N K D L T M L F M R T N O A N L G R C L E D I

1561 TGTTCGCGTGGGCTTCATTGACACCAACAGCATTGGCTGCGTCGCATCATCAGGCGTGCTTTATATCTCCCTCGTTTTCATTATCGGCGTCGTCGCCATTCGATTCATCATGGCCGTCAT 1681 GTTTCAATGGTTCTTCTCTTGGAAACTGGGCAACTTCCCTCGCGAAACTTATGAACAACGGATGGCAAGGTCGGCAGAAATTGAGAACTGGACAAGCGACATTTATCGCCCGGCGCCGAG
 1921 TATGTTGGATTCGTCTTCGTCCAACTGGAAGCGGTCTACCATGTATGCTGCATCGGCGAAAGGTGGGCAAAAAGGTGCCGAATCTCCAAGTCGATATTCTAGAAGCACTACGTCGTTCGG CGATTCTGCGAAAGGAGGATTTTTGGACAACCCTTGTCCTTTCCCATTGCACAATGTCGTСССССАGССGССGССАGAСTTCGAGCCATTCAACTTCCCTCTTGCCCATACAATTTGCCT
 2161 CGTGACGGCCTACTCAGAATCCGTGGAAGGATTGAGAACAACTCTCGACTCACTTGCGACCACCGACTACCCCAATAGCCACAAGTTGATCCTCATCATCGCTGACGGTATGGTCAAGGG

2281 TGCCGGTAATAACCTGACGACTCCGGAAATTTGCCTGACCATGATGAAGGAATTTGTCATTGCACCGAACGAGTTCGAACCCCACTCGTATGTCGCCACTGCAGACGGACACAAAAGACA
2401 CAACATGGCGAAGGTATATGCTGGATTTTTACGATTACGACAACGCAACGGTGGAGAGGTCGAAGCAACAACGTGTGCCAGTGGTGCTCGTCGCCAAGTGCGGTAATCCACTTGAAGCCAA


2521 CGACTCGAAACCTGGAAACAGAGGAAAGCGGGATAGCCAGATCGTCCTCATGGGATTCTTGCAAAAAGTTATGTTTGACGAACGAATGACAACGTTCGAGTATGAGTTCTTCAACTCGAT

2641 ATGGAGGGCCACGGGTGTTAGTCCGGATCGATATGAGTTGGTTCTGTGTGTCGATGCCGATACCAAGGTTTTCCCAGATTCCTTGACGAGGATGGTCAGCTGTATGGTTCACGATGAGGA


2761 GATCATGGGTCTTTGTGGAGAGACGAAGATTGCTAATAAGGCGGAGACCTTTGTTACCATGATGCAAGTCTTCGAATACTACATCTCGCACCACATGACGAAAGCCTTCGAGTCGATGTT I M G L C G E T K I A N K A E T F V T M M

3001 GAACGTTGTCGACACATTGCACAAGAAGAATCTCCTACTCCTCGGTGAAGATCGGTACCTCACGACTCTCATGCTCAAGACCTTCCCCAAACGCAAGAACATGTTCTGTCCGCAAGCTGT


3121 TTGCAAGACCGTTGTCCCCGACACGTTCCGCGTCCTTCTCTCCCAACGACGACGATGGATCAACTCCACTATCCACAATTTGGCCGAGTTGTTACTGGTTCGAGACCTGTGCGGCACGTT
3241 CTGTTTCTCGATGCAGTTCGTGGTAGGCATGGAGTTGGCTGGAACGCTTGTCCTACCGGCCGCTATAGCATTCACGTTGTACTTGATCATCGTTTCCATCATTCCGGGTGGGACAGATAC

3361 AACCATTCCACTCGTACTGCTCGCCGTCGTTCTTGGTCTGCCGGGTCTCCTTATCGTAGTCACTTCGCGGAAGATCGCCTACATTGGTTGGATGCTGGTGTACCTCCTCTCCTTGCCCAT
3481 CTGGAACGGTATACTGCCCGCGTACGCATTCTGGCACTTCGATGATTTCTCGTGGGGTCAGACGAGGCAGGTTGCTGGAGAGGACAAAGAGAACCATGGCGATAAGGAAGGAGAATTCGA

3601 CTCAACGCATATCGTCATGAAGAGGTGGGCAGAGTTTGAGCGAGATAGAAGGTGGAAGAGTGGCACTCAGTCCAGGGATTCGACCTTCTATGAAAGGAAgtgagtaataaggcctcagta


3721 tgagtatatggcgctgataagagtgttagGGCTGATAGTAACCGCTATTCTCTGGCTTCGAATTCGGATACTTGGCATCCTCCATCCAACGGTTTCGATACAAGCACTGTGGACTCTACG A $\quad \mathrm{D} \quad \mathrm{S} \quad \mathrm{N} \quad \mathrm{R} \quad \mathrm{Y}$

3961 AGTGAAGATCAGACCTACCAGTCCGACGTCGGCTCGACCTCCCATCAACGCCTTGTCCCCAGCCCCAAGTCCGGCGACCAATACAGCGACTCAATGGAATCCACTGTCTCTGGAAGTGTC

4081 GGTACTCCCGTACGATACTCTTCATCACCACATTACGAATCTCCCATTCCACGGATGCCTCCTAACGTCAACATCCGAACTGCCCCCGTGCAAACAGGATACGCGACGGAGGGGTACAAC
 4201 CCATTCGGTGGCTCCAGCGGCGGCAGTCTGGTTCAGTCGCCTGGAGGGTATGAGGAGAGTAGTGCATACAGTGCTGAAACGGAGGAAGTCAGCGGGTTCGCCGGTGTGGGCGCAGGGGA

4441 GCCCAGAATCGATACTCGCGTAACTCTAGTGCGTACAACCTCCCGCCAGGCGCTGCGCCGCCCCAACCATACCACGGTGGCTACAATTAACCTTCCTCTCGGCGAATTTTCTTCGGTCTC

4561 TСССТТСТGTTTTCATCACGCAAATACACAAGTACTTTTATGGCCTAATGAACGTCATATAACAATCTCCTGTTGCATACCTTTTCTCGTTTACGGACTACGTGTTGGCTCTCGCAACGT 4681 ACAGTACCTACCTCCGCCAAAACCTATCCGCTGTCTCTACTATTTCAGCCTCGGACGGACAACTTCGTTCATGTTCATATGACTGCATCCTTTCCTGTCTACTAGCTTGTTGCTCTCACA
4801 TCGAATGGACCTCTGGCTCTCTCTCCCCTCCATCACGCTTATTCACGATATATATCCCTACTACACTCGACGTTGCATTGCTCGCCTCTCCTTTATCATTAGCATCTGCTATTTAATGTC
4921 AACTCGCAAGTTGTCCTTAATGAGCCTTATCCTGAAATGTAACGCTGAGCGATTTCGAGACTGGCATTTTACAAGTACAACATTTCGGTAATAAGCCGACATAGATATATTAAACAAGCG
5041 ACACTCCATCGGAAACCAGACCCTACATGAGCGATGAGTAATTTGCCTACTAGTTGTCACGGATGGACTCACTTGGAGGGCTCCTGGGTTGGCACCAACAAGCTCCTTGATGA

PoChs1
UmChs5
NcChs4
GvChs3
AnChsD BcChsIV
CaCHS3
ScCHS3

PoChs1
UmChs 5
NcChs4
GvChs3
AnChsD
BcChsIV
CaCHS3
ScCHS3

PPPDFEPFNFPLAHTICLVTAYSESVEGLRTTLDSLATTDYPNSHKLILIIADGMVKGAGNNLTTPEICLTMMKEFVIAPNEFEPHSYVATADGHKRHNM PGPDYRPFGFQLAHSICLVTAYSESFEGLRTTLLDSLATTDYPNSHKLLLVIADGIVKGAGSDISTPDICLSMMKDLVIPAEEVEGNSYVAIADGYKRHNM PPSDWMPFGFPLAHTICLVTAYSEGEMGVRTTLDSIAMTDYPNSHKVILVICDGIIKGHGEEHSTPDIILGMMKDHTIHPDDVEPFSYVAVATGSKRHNM PPPEYQPFGFPLAHTMCLITCYSEGEQGVRSTLDSIATTDYPNSHKLMLIVCDGLVKGSGETLTTPEIILSMMKDHAVHPDDITPFSYVAVASGSKRHNM PPPDWQPYGFPLAHAMCLVTCYSEGEEGIRTTLDSIALTDYPNSHKSIVVICDGIIKGKGEEFSTPDIVLRMMRDPIIPPEEVEAFSYVAVATGSKRHNM PPKEWEPYGFPLAHAILLVTAYSEGELGIRTTLDSIATTDYPNSHKTILVICDGIIKGEGEPKATPEIVLGMMKDFVTPVDEVPAYSYVAVERGSKRHNM PPVEYQPFGYPLAHTINLVTCYSEDEEGIRITLDSIATTDYPNSHKLILVICDGIIKGSGNDETTPDIVLDMMSDLTVPRDEVEAYSYVAVAQGSKRHNM PPLDFMPYGFPLIHTICFVTCYSEDEEGLRTTLDSLSTTDYPNSHKLLMVVCDGLIKGSGNDKTTPEIALGMMDDFVTPPDEVKPYSYVAVASGSKRHNM AKVYAGFYDYDNATVER-SKQQRVPVVLVAKCGNPLEANDSKPGNRGKRDSQIVLMGFLQKVMFDERMTTFEYEFFNSIWRATGVSPDRYELVLCVDADT CKIYAGFYDYDDETVER-SKQQRVPMILVAKCGTPLEADSAKPGNRGKRDSQVLLMAFMQKVMFDERMTAFEYEFFNSIWRVTGVSPDNYEIVLCVDADT AKVYTGFYDYGTNSAIPLEKQQRVPMMMVVKCGTPAEASKSKPGNRGKRDSQIILMSFLQKVMFDERMTELEYEMFNGLWKITGISPDFYEIVLMVDADT AQVYAGFYDYGEQSMVPVEKQQRVPMLVVVKCGTPAEKSAKKPGNRGKRDSQIILMSFLQKVMFDERMTELEYEMFNGIWKVTGISPDYYETVLMVDADT AKVYAGFYDYGEHSIIPVEKQQRVPMMIIVKCGTPAEATAAKPGNRGKRDSQIILMSFLQKVMFDERMTELEYEMFNGLLHVTGIPPDFYEVVLMVDADT AKVYAGFYDYGADSTINVNRQLRVPMVCIVKCGTPDEATHRKPGNRGKRDSQIILMSFLQKVMFDERMTELEFEIFNGFYQISGIFPDMYEVVLMVDADT AKVYAGFYKYN-DETVPPEKQQRIPMITIVKCGTPEEASAPKPGNRGKRDSQIILMSFLQKVVFDERMTSLEYEMLQSIWRITGLMAEFYEIVLMVDADT AKIYAGFYKYD-DSTIPPENQQRVPIITIVKCGTPAEQGAAKPGNRGKRDSQIILMSFLEKITFDERMTQLEFQLLKNIWQITGLMADFYETVLMVDADT

[^1]PoChs 1
UmChs 5 NcChs 4 GvChs 3 AnchsD BcChsIV CaCHS3 ScCHS 3

KVFPDSLTRMVSCMVHDEEIMGLCGETKIANKAETFVTMMQVFEYYISHHMTKAFESMFGGVTCLPGCFSMYRIKAPKGDDGYVVPILANPDIVEHYSEN KVFPDSLSRMVACMVEDPEIMGLCGETKIANKSETWVTMIQVFEYYISHHQTKAFEACFGGVTCLPGCFSAYRIKAPKGPHGYWVPILANPDIVEHYSEN KVFPDSLTHMISAMVKDPEIMGLCGETKIANKRASWVSAIQVFEYFVSHHLAKAFESVFGGVTCLPGCFCMYRIKAPKGAQNYWVPILANPDVVEHYSEN KVFPDSLTHMVGAMVHDPEIMGLCGETKIANKRTSFVSMMQVFEYFISHHQTKAFESVFGGVTCLPGCFCMYRIKAPKGGQNYWVPILANPDVVEHYSED KVFPDSLTHMISAMVKDPEVMGLCGETKIANKTDSWVTMIQVFEYFVSHHQSKAFESVFGGVTCLPGCFSMYRIKAPKGGQNYWVPILANPDIVEHYSEN KVFPDSLTHMVSAMVKDPEIMGLCGETKIANKRASFTTAIQVFEYFISHHSVKSFESIFGGVTCLPGCFSMYRIKAPKGGN-YWVPILANPDVVEHYSDN KVFPDSLTHMVAEMVKDPTIMGLCGETKISNKAQTWVTAIQVFEYYISHHQAKAFESIFGGVTCLPGCFCMYRIKAPKGSDGYWVPILANPDIVERYSDN KVFPDALTHMVAEMVKDPLIMGLCGETKIANKAQSWVTAIQVFEYYISHHQAKAFESVFGSVTCLPGCFSMYRIKSPKGSDGYWVPVLANPDIVERYSDN


VVDTLHKKNLLLLGEDRYLTTLMLKTFPKRKNMFCPQAVCKTVVPDTFRVLLSQRRRWINSTIHNLAELLLVRDLCGTFCFSMQFVVGMELAGTLVLPAA 1045 VVDTLHKKNLLLLGEDRYLTTLMLKTFPKRKMMFVPQAVCKTIVPDTFRILLSQRRRWINSTVHNLFELVMVNDLCGTFCFSMRFVVFMELTGTLVLPAA 1061 VVDTLHKKNLLLLGEDRYLSTLMLRTFPKRKQVFVPQAVCKTTVPDEFMVLLSQRRRWINSTIHNLMELVLVRDLCGTFCFSMQFIVGIELIGTLVLPAA 1034 VVDTLHTKNLLLLGEDRYLSTLMLKTFPKRKQIFVPQAVCKTIVPDSFGVLLSQRRRWINSTVHNLMELVLVRDLCGTFCFSMQFVVFIELIGTVVLPAA 1077 VVDTLHKKNLLLLGEDRYLSTLMLRTFPKRKQIFVPQAVCKTVVPDKFMVLLSQRRRWINSTVHNLMELVLVRDLCGTFCFSMQFVIFVELVGTVVLPAA 1054 VVDTLHKKNLLLLGEDRYLSTLMLRTFPKRKQVFVPQAVCKTQVPDSLWILMSQRRRWINSTVHNLMELVLVRDLCGTFCFSMQFVVFIDLVSTLVLPAA 1070 VVDTLHRKNLLLLGEDRYLSSLMLRTFPTRKQVFVPKAACKTVVPDKFKVLLSQRRRWINSTVHNLFELVLVKDLCGTFCFSMQFVIFIELIGTLVLPAA 1048 VTNTLHKKNLLLLGEDRFLSSLMLKTFPKRKQVFVPKAACKTIAPDKFKVLLSQRRRWINSTVHNLFELVLIRDLCGTFCFSMQFVIGIELIGTMVLPLA 1038

PoChs1
UmChs5 NcChs 4 GvChs 3 AnChsD BcChsIV
CaCHS3
ScCHS3 * *** ************** *** **********

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 Pochs 1 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 ( $M Y$ ), pileus of immature fruit body (IP), stipe of immature fruit body (IS), pileus of mature fruit body (MP), and stipe of mature fruit body $(M S)$. B Comparison of relative expression levels of Pochsl between mycelia with $(+)$ and without (-) the temperature-downshift treatment

A


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

This result led us to examine the effect of the tempera-ture-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 Pochsl in mycelia. The mycelia were grown on the saw-dust-based medium already described at $25^{\circ} \mathrm{C}$ in the dark for 10 days, and subsequently the culture was continued for an additional 5 days, either at $15^{\circ} \mathrm{C}$ in the dark (the tem-perature-downshift treatment: + ) or at $25^{\circ} \mathrm{C}$ 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 fruitbody 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 Pochsl expression seems to be interesting and is now underway. An increase (1.9 fold) in the expression level of a gene encoding $\Delta 9$ 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.

## References

Bartnicki-García S (1968) Cell wall, chemistry, morphogenesis and taxonomy of fungi. Annu Rev Microbiol 22:87-108

Bowen AR, Chen-Wu JL, Momany M, Young R, Szaniszlo PJ, Robbins PW (1992) Classification of fungal chitin synthases. Proc Natl Acad Sci U S A 89:519-523
Bulawa CE (1993) Genetics and molecular biology of chitin synthesis in fungi. Annu Rev Microbiol 47:505-534
Chen-Wu J, Zwicher J, Bowen AR, Robbins PW (1992) Expression of chitin synthase genes during yeast and hyphal growth phases of Candida albicans. Mol Microbiol 6:497-502
Chigira Y, Abe K, Gomi K, Nakajima T (2002) chsZ, a gene for a novel class of chitin synthase from Aspergillus oryzae. Curr Genet 41:261-267
Choi WJ, Santos B, Durán A, Cabib E (1994) Are yeast chitin synthases regulated at the transcriptional or the posttranslational level? Mol Cell Biol 14:7685-7694
Durán A, Bowers B, Cabib E (1975) Chitin synthetase zymogen is attached to the yeast plasma membrane. Proc Natl Acad Sci U S A 72:3952-3955
Ichinomiya M, Motoyama T, Fujiwara M, Takagi M, Horiuchi H, Ohta A (2002) Repression of chsB expression reveals the functional importance of class IV chitin synthase gene chsD in hyphal growth and conidiation of Aspergillus nidulans. Microbiology 148:13351347
Klimyuk VI, Carroll BJ, Thomas CM, Jones JD (1993) Alkali treatment for rapid preparation of plant material for reliable PCR analysis. Plant J 3:493-494
Lerner MR, Boyle JA, Mount SM, Wolin SL, Steitz JA (1980) Are snRNPs involved in splicing? Nature (Lond) 283:220-224
Liu YG, Whittier RF (1995) Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics 25:674-681
Motoyama T, Sudoh M, Horiuchi H, Ohta A, Takagi M (1994) Isolation and characterization of two chitin synthase genes of Rhizopus oligosporus. Biosci Biotechnol Biochem 58:1685-1693
Müller C, Hjort CM, Hansen K, Nielsen J (2002) Altering the expression of two chitin synthase genes differentially affects the growth and morphology of Aspergillus oryzae. Microbiology 148:40254033
Munro CA, Schofield DA, Gooday GW, Gow NAR (1998) Regulation of chitin synthesis during dimorphic growth of Candida albicans. Microbiology 144:391-401
Nagahashi S, Sudoh M, Ono N, Sawada R, Yamaguchi E, Uchida Y, Mio T, Takagi M, Arisawa M, Yamada-Okabe H (1995) Characterization of chitin synthase 2 of Saccharomyces cerevisiae: implication of two highly conserved domains as possible catalytic sites. J Biol Chem 270:13961-13967
Niño-Vega GA, Carrero L, San-Blas G (2004) Isolation of the CHS4 gene of Paracoccidioides brasiliensis and its accommodation in a new class of chitin synthases. Med Mycol 42:51-57
Pammer M, Briza P, Ellinger A, Schuster T, Stucka R, Feldmann H, Breitenbach M (1992) DIT101 (CSD2, CAL1), a cell cycle-regulated yeast gene required for synthesis of chitin in cell walls and chitosan in spore walls. Yeast 8:1089-1099
Roncero C (2002) The genetic complexity of chitin synthesis in fungi. Curr Genet 41:367-378
Ruiz-Herrera J, González-Prieto JM, Ruiz-Medrano R (2002) Evolution and phylogenetic relationships of chitin synthases from yeasts and fungi. FEMS Yeast Res 1:247-256
Sakai H, Kajiwara S (2003) A stearoyl-CoA-specific $\Delta 9$ fatty acid desaturase from the basidiomycetes Lentinula edodes. Biosci Biotechnol Biochem 67:2431-2437
Specht CA, Liu Y, Robbins PW, Bulawa CE, Iartechouk N, Winter KR, Riggle PJ, Rhodes JC, Dodge CL, Culp DW, Borgia PT (1996) The chsD and chsE genes of Aspergillus nidulans and their roles in chitin synthesis. Fungal Genet Biol 20:153-167
Sreenivasaprasad S, Burton KS, Wood DA (2000) Cloning and characterization of a chitin synthase gene cDNA from the cultivated mushroom Agaricus bisporus and its expression during morphogenesis. FEMS Microbiol Lett 189:73-77
Sudoh M, Nagahashi S, Doi M, Ohta A, Takagi M, Arisawa M (1993) Cloning of the chitin synthase 3 gene from Candida albicans and its expression during yeast-hyphal transition. Mol Gen Genet 241: 351-358
Takeshita N, Ohta A, Horiuchi H (2002) $\operatorname{csm} A$, a gene encoding a class V chitin synthase with a myosin motor-like domain of Aspergillus
nidulans, is translated as a single polypeptide and regulated in response to osmotic conditions. Biochem Biophys Res Commun 298:103-109
Takeshita N, Ohta A, Horiuchi H (2005) CsmA, class V chitin synthase with a myosin motor-like domain, is localized through direct interaction with actin cytoskeleton in Aspergillus nidulans. Mol Biol Cell 16:1961-1970
Takeshita N, Yamashita S, Ohta A, Horiuchi H (2006) Aspergillus nidulans class V and VI chitin synthases CsmA and CsmB, each with a myosin motor-like domain, perform compensatory functions that are essential for hyphal tip growth. Mol Microbiol 59:13801394
Wang Z, Szaniszlo PJ (2000) WdCHS3, a gene that encodes a class III chitin synthase in Wangiella (Exophiala) dermatitidis, is expressed differentially under stress conditions. J Bacteriol 182:874-881

Wang Q, Liu H, Szaniszlo PJ (2002) Compensatory expression of five chitin synthase genes, a response to stress stimuli, in Wangiella (Exophiala) dermatitidis, a melanized fungal pathogen of humans. Microbiology 148:2811-2817
Xoconostle-Cázares B, Specht CA, Robbins PW, Liu Y, León C, RuizHerrera J (1997) Umchs5, a gene coding for a class IV chitin synthase in Ustilago maydis. Fungal Genet Biol 22:199-208
Yabe T, Yamada-Okabe T, Nakajima T, Sudoh M, Arisawa M (1998) Mutational analysis of chitin synthase 2 of Saccharomyces cerevisiae. Eur J Biochem 258:941-947
Yamazaki T, Kiyofuji T, Johjima T, Kajiwara S, Tsukamoto A, Sugiura J, Shishido K (2004) Isolation of a ras gene from the basidiomycete Coriolus hirsutus and use of its promoter for the expression of Pleurotus ostreatus manganese (II) peroxidase cDNA in C. hirsutus. Mycoscience 45:317-323


[^0]:    M. Nishihara • A. Watanabe • Y. Asada ( $\boxtimes$ )

    Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan
    Tel. +81-87-891-3112; Fax +81-87-891-3021
    e-mail: asaday@ag.kagawa-u.ac.jp

[^1]:    * *** *

