FULL PAPER

Mikihiro Nishihara · Akira Watanabe · Yasuhiko Asada

Isolation and characterization of the gene encoding a chitin synthase with a myosin motor-like domain from the edible basidiomycetous mushroom, *Lentinula edodes*, and its expression in the course of fruit-body formation

Abstract We isolated and characterized the genomic and complementary DNAs encoding a chitin synthase from an edible basidiomycetous mushroom, *Lentinula edodes*. The gene (which we designated *Lechs1*) contains a large open reading frame encoding a polypeptide of 1937 amino acid residues. The open reading frame is interrupted by 14 small introns (49–116 bp). The gene product (LeChs1) consists of a myosin motor-like domain in its N-terminal half and a chitin synthase domain in its C-terminal half, analogous to the class V and VI chitin synthases of other filamentous fungi. Phylogenetic analysis demonstrated that LeChs1 is classified into class VI chitin synthases. Southern blot analysis indicated that *Lechs1* is a single-copy gene per haploid genome and that *L. edodes* has no other highly homologous chitin synthase genes. Northern blot analysis revealed that *Lechs1* is expressed throughout the whole stages of fruit-body formation of *L. edodes*, but its expression level gradually declines in a fruit body-maturation-dependent manner with highest expression in vegetative mycelia and fruit body at the early stage of maturation (immature fruit body). This is the first report on the isolation and characterization of the gene encoding a chitin synthase with a myosin motor-like domain from basidiomycetes.

Key words Basidiomycetous mushroom · Chitin synthase · Fruit body · *Lentinula edodes* · Morphogenesis

Introduction

Basidiomycetous mushrooms are unique microorganisms that undergo a dramatic morphological differentiation from vegetative mycelia to a fruit body. The molecular mechanism of the process of the fruit-body formation, a very important and attractive topic for study from both scientific and commercial aspects, remains largely unknown. The basic points in fruit-body formation of basidiomycetes have been defined as hyphal knot formation, formation of initial aggregate, differentiation of the primordium with distinct pileus (cap) and stipe tissues, fruit-body maturation by cell extension, and basidiospore formation (Kües and Liu 2000). Through the process of the fruit-body formation in basidiomycetes, cell wall components have to be synthesized, degraded, and reconstituted precisely.

Chitin, a β-1,4-linked homopolymer of *N*-acetylglucosamine (GlcNAc), is one of the major structural components of the cell walls of a wide range of fungi and contributes to cell wall structural rigidity and osmotic integrity (Smits et al. 1999; Sassoon and Mooibroek 2001; Ruiz-Herrera et al. 2002). The regulation of its metabolism is known to be important for fungal morphogenesis (Bulawa 1993; Cid et al. 1995). The polysaccharide chitin is synthesized by chitin synthases (EC 2.4.1.16), which are membrane-bound proteins catalyzing the polymerization of GlcNAc using UDP-GlcNAc as a substrate. Fungi have 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). Among them, classes III, V, and VI have been found only from filamentous fungi (Bowen et al. 1992), suggesting that these isozymes play a unique role in hyphal growth. The roles of chitin synthases in the determination of morphology have been analyzed 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 basidiomycetes. To study the roles of chitin synthesis in the morphogenesis of basidiomycetous mushrooms, we isolated and characterized a chitin synthase gene from an edible basidiomycetous mushroom, *Lentinula edodes* (Berk.) Pegler, and analyzed its expression pattern during fruit-body formation. *L. edodes*, known as the Shiitake mushroom, is one of the most important cultivated edible mushrooms in commercial production, second only to *Agaricus bisporus* (J.E. Lange) Pilát, in the world (Kües and Liu 2000).

M. Nishihara · A. Watanabe · Y. Asada (✉)
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

Materials and methods

Strains, media, and culture conditions

The commercial *Lentinula edodes* strain (SB1226, dikaryon) was used in this study. The vegetative mycelia were grown at 25°C on potato dextrose agar (PDA) medium (Becton Dickinson, Baltimore, MD, USA) in the dark. Fresh fruit bodies of *L. edodes* at different maturation stages were kindly provided by mushroom producer Shikoku Biotech (Kagawa, Japan). For fruit-body production, vegetative mycelia were inoculated into sawdust medium containing rice bran (sawdust : rice bran, 1 : 1 w/w) and grown for 4 weeks at 25°C in the dark. The fruit bodies were induced by submerging the cultures into water for 3 h and then transferring into continuous illumination at 15°C, with the humidity at 90% (fruiting treatment), for 3 weeks. *Escherichia coli* (Migula) JM109 (Yanisch-Perron et al. 1985), used for subcloning and plasmid preparation, was grown in Luria–Bertani (LB) medium (Bertani 1951).

PCR amplification and sequencing of *Lechs1*

Lentinula edodes genomic DNA was prepared by the cetyltrimethylammonium bromide (CTAB) method (Klimyuk et al. 1993) from vegetative mycelia frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle.

To obtain chitin synthase gene fragments, polymerase chain reaction (PCR) amplifications were run with the *L. edodes* genomic DNA as a template and the following two primer pairs. For amplification of parts of class I–III chitin synthase genes, the primer pair of F1 (5′-CTGAAGCT TACNATGTAYA AYGARGA-3′) and R1 (5′-GTTCTC GAGYYTTRTAYTCRAARTTYTG-3′) designed according to Bowen et al. (1992) was used. For amplification of parts of class IV–VI chitin synthase genes, the primer pair of F2 (5′-CCTGGAAACCGTGGCAAGCGTG-3′) and R2 (5′-AGAGTAGTGAGATAACGATCTTCTCC-3′) was used. The primers F2 and R2 were designed on the basis of highly conserved sequences, PGNRGKRD and GEDRYLTTL, respectively, among the fungal class IV–VI chitin synthases, and also on the basis of the codon usage for *L. edodes* genes. PCR amplifications were carried out under standard conditions using a Thermal Cycler (Takara Bio, Otsu, Japan) and *Takara ExTaq* polymerase (Takara Bio). The amplified genomic DNA fragments were gel-purified using the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) following the manufacturer's instructions, cloned using T/A strategy into the pGEM-T Easy vector (Promega, Madison, WI, USA), and then sequenced.

To obtain the complete sequence of one (hereafter designated *Lechs1*) of the *L. edodes* chitin synthase genes, the thermal asymmetric interlaced (TAIL) PCR technique (Liu and Whittier 1995) was employed as follows. The specific primers for TAIL-PCR were designed based upon the nu-cleotide sequence of the aforementioned genomic DNA fragment, which was PCR-amplified with the primer pair of F2 and R2. For amplification of the 5′-upstream genomic region of *Lechs1*, the specific primers R3 (5′-TCTTGT GAAGGGTATCAACGTTGGG-3′), R4 (5′-GGTATCA ACGTTGGGGTTCGGCGTAC-3′), and R5 (5′-GTCGAT AGCGGCCGAAGCTACAAGC-3′) were used in three consecutive PCRs in combination with an arbitrary degenerate primer AD1 (5′-NGTCGASWGANAWGAA-3′), according to the conditions reported by Liu and Whittier (1995). Similarly, the specific primers F3 (5′-AACCGT GTTCACTTTGACGCGCC-3′), F4 (5′-CGCTAGAGCT GGAGCTGTATCATC-3′), and F5 (5′-GTGACACCAG AATCTCTCAACAGGC-3′) were used with an arbitrary AD2 primer (5′-GTNCGASWCANAWGAA-3′) for amplification of the 3′-downstream genomic region of *Lechs1*. The amplified genomic DNA fragments were cloned into the pGEM-T Easy vector and then sequenced. To amplify full-length genomic DNA including the open reading frame and the 5′- and 3′-flanking regions of *Lechs1*, two primers (F6; 5′-CCTTGGCGATTGGGCCGCGTCG-3′ and R6; 5′-AACGAATGAAAGGGTCTATATTCTATTCC-3′) were synthesized according to the sequences that became known by the TAIL-PCR mentioned above. The amplified genomic DNA fragment (*Lechs1*) by PCR performed with *L. edodes* genomic DNA as a template and primers mentioned above (F6 and R6) was gel-purified and then sequenced. The sequence was confirmed by repeating sequence reactions using the uncloned PCR-amplified DNA fragment as a template to exclude mistakes resulting from the use of PCR.

RT-PCR for amplification of cDNA corresponding to *Lechs1*

The total RNA was isolated from the *L. edodes* vegetative mycelia using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as in the manufacturer's instructions. The RNA sample was treated with RNase-free DNaseI (Takara Bio) to remove contaminating DNA.

To confirm the translation initiation codon of the *Lechs1* gene 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. The gene specific antisense primer (R7; 5′-CCTCTCCGTCGATATCACTGTGTTG-3′) used for 5′-RACE was designed from the sequence of *Lechs1* mentioned previously. The 5′-RACE product was gel-purified and then sequenced. To determine the complete sequence of *Lechs1* cDNA, the DNA fragments of cDNA were amplified by reverse transcriptase-PCR (RT-PCR) using several specific primers designed based on the *Lechs1* sequence and then sequenced. Reverse transcription was carried out using SuperScript III RNase H⁻ reverse transcriptase (Invitrogen) following the manufacturer's instructions.

Determination and analyses of nucleotide sequences

DNA sequencing was performed using BigDye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA, USA) on ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The database comparison was made at the GenomeNet www server using BLAST2 program (http://blast.genome.jp/). Alignment was generated using the ClustalW program (Thompson et al. 1994).

Southern and Northern blot analyses

Southern and Northern blot analyses were carried out using ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare Bio-Sciences) according to the manufacturer's instructions. A partial DNA fragment of *Lechs1* amplified by PCR (1070 bp, nucleotides 4098 to 5167 in Fig. 1) was labeled and used as a probe.

For Southern blot analysis, genomic DNA of *L. edodes* was digested with *Eco*RI, *Pvu*II, or *Sal*I, separated on a 0.8% agarose gel, transferred onto a nylon membrane (Pall, East Hills, NY, USA), and then probed.

For Northern blot analysis, total RNA samples were prepared from vegetative mycelia and fruit bodies at different stages of maturation (stages I–III) using TRIzol Reagent as mentioned previously. Each fruit body was separated into stipe and pileus before RNA isolation. The RNA samples (30 μg each) were separated on 3.2 M formaldehyde–0.8% agarose gel, transferred onto a nylon membrane, and then probed. Both analyses were performed under stringent hybridization conditions.

Results and discussion

Identification of chitin synthase gene homologues in the *Lentinula edodes* genome

To identify the chitin synthase gene homologues in the *L. edodes* genome, PCR experiments were carried out using *L. edodes* genomic DNA as a template and two pairs of primers (F1 + R1 and F2 + R2; see Materials and methods). Three distinct DNA fragments of approximately 300, 450, and 850 bp, respectively, were amplified using the primer pair of F1 + R1. The sequence analysis of the PCR products revealed that 300- and 450-bp amplicons were nonspecific; however, 850-bp DNA fragment encoded a polypeptide with high homology to other fungal class II chitin synthases (data not shown). On the other hand, a single PCR product (approximately 600 bp) was amplified by using the primer pair of F2 + R2. The sequence analysis demonstrated that the PCR product also encoded a polypeptide highly homologous to other fungal chitin synthases, indicating that the PCR product was presumed to derive from the *L. edodes* chitin synthase gene (we designated this *Lechs1*). These results suggested that *L. edodes* has a multiple chitin synthase gene family in the genome, as reported previously for other fungi (Munro and Gow 2001; Roncero 2002).

Sequencing and characterization of *Lechs1*

To determine the whole coding sequence and 5′- and 3′- flanking sequences of *Lechs1*, we employed the TAIL-PCR method (see Materials and methods). A 7691-bp region was sequenced on both strands (see Fig. 1). In the process of the nucleotide sequence determination by direct sequencing, we detected no ambiguities and errors arising from the existence of the allele with a different sequence. This result may be explained as follows. Some commercially cultivated mushroom strains (dikaryon), such as the *L. edodes* strain we used in the study, are assumed to have few allelic differences in the genomes from the point of view of their breeding background of repeated selfing. We also determined the whole coding sequence of cDNA corresponding to the *Lechs1* gene. A comparison between these sequences revealed that the *Lechs1* gene contains a large open reading frame of 6670 bp interrupted by 14 small (49–116 bp) introns and encodes a polypeptide of 1937 amino acid residues with a calculated molecular mass of 215 kDa and an isoelectric point of 5.33. The genomic DNA and cDNA sequences of *Lechs1* will appear in the DDBJ/EMBL/GenBank databases under the accession numbers AB262360 and AB262359, respectively. The presence of many small introns is the characteristic feature of the genes of basidiomycetous fungi. All 14 introns conform to the universal GT/AG splicing rule (Lerner et al. 1980) observed for typical splicing sites at intron–exon junctions. The 5′-RACE experiment showed that the RACE product initiated at 51 nucleotides upstream from the translation start codon, suggesting that this position may be the transcription starting point of *Lechs1*. The promoter region of *Lechs1* contains CAAT box (CCAAT, at position −143 to −139 relative to the translation start codon) to which the HAP complex binds (Litzka et al. 1998; Steidl et al. 1999) and two TATA box-like sequences (TATACTAT and TATATGA, at positions −185 to −178 and −81 to −75, respectively). Also, we found a regulatory element, the Stunted protein (StuAp) response element (GTGCGCGTTG at position −424 to −415), in the promoter region. StuAp is a member of a family of fungal transcription factors (APSES proteins) that have been shown to play an important role in controlling fungal morphogenesis and development (Dutton et al. 1997; Wu and Miller 1997; Doedt et al. 2004; Sheppard et al. 2005), implying the possibility that *Lechs1* is regulated and plays a certain role in the morphogenesis (fruit-body formation) of *L. edodes*. Two polyadenylation signal-like sequences (AATAG<u>AA</u> and <u>AA</u>TATA, in which underlined A nucleotides overlap) were found in the 3′-flanking region.

The results of an InterProScan analysis (http://www.ebi. ac.uk/ InterProScan/) of the *Lechs1* gene product (LeChs1) demonstrated that LeChs1 contains a myosin motor-like domain (MMD) (Fujiwara et al. 1997) in the N-terminal region (comprising approximately 603 amino acids, amino acid residues 19 to 621) and a chitin synthase domain (CSD) (Fujiwara et al. 1997) in the C-terminal region (comprising approximately 525 amino acids, amino acid residues 1228 to 1752). The genes encoding chitin synthases with an N-

Fig. 1. Nucleotide sequence of *Lechs1* and deduced amino acid sequence. The predicted transcription starting point is shown by an *arrow*. Introns are indicated by *lowercase letters*. The putative StuAp response element, CAAT box, two TATA box-like sequences, and two overlapping polyadenylation signal-like sequences are *underlined*. The putative consensus motifs of myosins (P-loop and switch II) are indicated by *boldface letters*. Two highly conserved domains that are implied as essential catalytic sites of chitin synthases are *boxed*

```
-915                                                             CCTTGGCGATTGGGCCGCGTCGCATGCTCCGGCGCATGGCGGCGCGGGAATTCGATTGTCCGAGTCATATGAATA
-840  GCGAGGGTAAAACTTTGATGATGCAGCGATGTACCACATAGATAGGATGATTGAACGAGCAGTGTGGCACGTTCTTCAACACCACCAACGAAAACAGACGCAGAGAGAGAGATGTTTTTT
-720  TTTCGAAGCGACGTAGACACTGAAATGTTGCCAGTGATTAGCCACTAGATCAAGAGAGAAAGATCGCCGAAATGCAGAGTAAGCGGAAAGAAGAGTTAGAACATACCTCTGACATACCTC
-600  ATGATTAGACCCATTCTAGTGACTTTTCCTGTTCCCAAAACAGTTGGTGAGCTTTTCTTCCCACCTTACGGTAACTGCCAAAAATGCATGGATGTGGGTCTGTACGACCGGATAGCACTG
-480  GCAGAGTTATTCTGTCATATGCCGAAGATTTTGCCGAGCATAATAATGCCGTTGATCCGTTCAGAAACGATTCCGAATTGAAAAGACATTGTCGGCGTGCGCGTTGCCAGTGGTCCGGTG
                                                                                                StuAp response element
-360  TCACAAGTCGACATTTTCCGCGACGACTCGGCCTCACCTTTTTCGATAATTCACCTACTCCCTTTCCTGCTGCTACATCTCTTCCAAGAAACGAATCCTGAACAATACCAAAGATAGCTG
-240  CTGGATCAACGAAACGGAAAGAGGCCATCAGAACTGCAGAATAAATAGACTCTAATATACTATACTCACGTCCGTACATTTTGATACTTGCTCAAGTCCAATGGCCATGACTTAGTGCGA
                                                            TATATACTATA                              CCAAT
                                                            TATA box                                CCAAT box
-120  AAAAAAGCAGGTCGACTAGGAGATAGTCCGGTATGTACATATATGAGTAGCCCGGATTCGAAATAACTGGCAGTGAACACGGTAATACTAATAATACCGTTGCAGTGACGCCACGTGGGA
                               TATATGAG                                     ↑ predicted transcription starting point
                               TATA box
  1   ATGAATTGGCCgtaggctgtctgcgtgtgtaacaattatttcttcttcctctcccactcgctgctacacaacaccaaggcagCATTTGCTGTTTGTTCCCTACCATCTCTCCTTTACGgtca
      M  N  W  P                                                                       I  C  C  L  F  P  T  I  S  P  Y  A
121   tggcctcaaatcacaatgtgcaggccgtacctctggcgacctcattggaccttgtttcgtcttccggctcagCGACCATCTATCCAACCGACGACGCTATTCTGGCTGTTTTACAAGCCA
                                                                           T  I  Y  P  T  D  D  A  I  L  A  V  L  Q  A  R
241   GGTTAGAGCTGATTTACCGTATACTCGCGCCGGAGCCACCAATTTTGTCGCAATTAATCCATATAAAACTCTAGCGAGTGTTAATAATGCCAGTGCAAAGGAATACGAGGAACGATCGT
      R  F  R  A  D  L  P  Y  T  R  A  G  A  T  N  F  V  A  I  N  P  Y  K  T  L  A  S  V  N  N  A  S  A  K  E  Y  E  E  R  S  Y
361   ATAAAGACACTAGTTTACCAATGGTTGACTCACCAAAGCTTCTACAGCCACATATTTACGACCTTGCTGCGAGGATGTATCTAGTGATGAGGAGAAGGAATGAGTCGCAGGCATTGCTCG
      K  D  T  S  L  P  M  V  D  S  P  K  L  L  Q  P  H  I  Y  D  L  A  A  R  M  Y  L  V  M  R  R  R  N  E  S  Q  A  L  L  A
481   CGAGgtgagcatgatcaacccacggcgcctcatttcttttatgctgagtttttcttttgcagGGGTATCACAGGCTCTGGAAAGACTTCAAATCTTCGTCTTTTTACCAACCAGATCTCAGG
      R                                                             G  I  T  G  S  G  K  T  S  N  L  R  L  F  T  N  Q  I  L  R
                                                                    P-loop
601   CTGTCCAGTCATTCGAGCAAAGAAGCCAAAATCGCAGAGCAAGTCAAAGCACTTGGAATCGTTCTCGAATCATTTGGTAACTCGAAAACGCTCATGAACCCAAATGCTTCACGACACAGT
      L  S  S  H  S  S  K  E  A  K  I  A  E  Q  V  K  A  L  G  I  V  L  E  S  F  G  N  S  K  T  L  M  N  P  N  A  S  R  H  S
721   CGATATACTGAGCTGCATTTCAACGAGCGAGGAAGAATTGCTGGTGCCAAAGTCCTCACTTTCGGACTCGACAAGTCTAGACTCAATCGCCTTACTCAGGAAGAACGGTCTTTTCACGTC
      R  Y  T  E  L  H  F  N  E  R  G  R  I  A  G  A  K  V  L  T  F  G  L  D  K  S  R  L  N  R  L  T  Q  E  E  R  S  F  H  V
841   TTCTATCAATTCATTGCTGGATGTACGTCCGCTGAACGAGATACACTCAATATTGAGGATCCCTCGGACTATGCCCTGTTAGCTTCTTCGGGTACCTACAGGCTTCCCGCGGGTCCGTCA
      F  Y  Q  F  I  A  G  C  T  S  A  E  R  D  T  L  N  I  E  D  P  S  D  Y  A  L  L  A  S  S  G  T  Y  R  L  P  A  G  P  S
961   AGCGATGACTCGGTTGCCATGGGTGACCTGCGAGCTGCTATGCGAACGCTTGGGTTCAAACCAAAAGCTACCGCAGCCATTTTTAGCCTCCTTATAGCCATTCTTCTTCTCGGCAACCTC
      S  D  D  S  V  A  M  G  D  L  R  A  A  M  R  T  L  G  F  K  P  K  A  T  A  A  I  F  S  L  L  I  A  I  L  L  L  G  N  L
1081  GAATTTGGTGAAGGTGATTTTCACACCGTATCCGCTCACGTTACCAATGTCGAGGTGCTCGACACGTATCTCGTCTGCTCGGAGTCTCCTCCGAAGACCTCTCGCAGGCTCTGACGAAC
      E  F  G  E  G  D  F  H  T  V  S  A  H  V  T  N  V  E  V  L  D  T  Y  L  V  C  S  R  L  L  G  V  S  S  E  D  L  S  Q  A  L  T  N
1201  AAGACGAGCTATGTGCGCAAGGAACTTTATACCGCTTTGCTGAACGCGCAGCAGAGTGCTCTTCAACGAGACCAGCTTGTCCGGGATCTCTATGCCATCATGTTCGCATTTGTTGTCGAA
      K  T  S  Y  V  R  K  E  L  Y  T  A  L  L  N  A  Q  Q  S  A  L  Q  R  D  Q  L  V  R  D  L  Y  A  I  M  F  A  F  V  V  E
1321  ACAGCGAACCCACAAGGTGCTCCGTCATCCAAATCACCCCCTCCGGACAGATTGTTTTGCTCGACCCAACCCGGGTATCAGTCTCGCGGTCCAACCGGAACAAGTTCCGTCGCCTCTT
      T  A  N  P  Q  G  A  P  S  S  K  S  P  P  P  H  S  Q  I  V  L  L  D  Q  P  G  Y  Q  S  R  G  P  T  G  T  S  S  V  A  L
                                                                    switch II
1441  TCGGGTAATGCTCCTCTCATATCCGCTTACGGCCAAAACGGCTTTGACGAGTTTTTGCATCAATTTTGCCGATGAGATGCTCCAGTCGTACTTCACGCGCAAACGTTTGAGGACACCGTT
      S  G  N  A  P  L  I  S  A  Y  G  Q  N  G  F  D  E  F  C  I  N  F  A  D  E  M  L  Q  S  Y  F  T  R  Q  T  F  E  D  T  V
1561  GGGTATAGTAATCATATTGTCAGCGACGGCGTGTCGATCCCCAGCGATATCAACAATGGATAATACGGCGTGTGTGGAGATGCTTCGAGGGCAGTTGCCGGATAAGGCGCAGCGGAAGCCT
      G  Y  S  N  H  I  V  S  D  G  V  S  I  P  A  I  S  T  M  D  N  T  A  C  V  E  M  L  R  G  Q  L  P  D  K  A  Q  R  K  P
1681  GGAGGGCTCTTGTCGCTGATGAACAAGGCTTCGTCTGCTCACAAGCAGGGTAAGGGTAACAGTGATCATCGTAATGAAGATTTGTTGCAGGAAATGCAAGCTAAATTCGGCGTGCATGCA
      G  G  L  L  S  L  M  N  K  A  S  S  A  H  K  Q  G  K  G  N  S  D  H  R  N  E  D  L  L  Q  E  M  Q  A  K  F  G  V  H  A
1801  TCCTTCGTCGCTACTTCTGGTAACGCAAACCGTATGCAGTTTGGTGTCAACCACTATGCTGGTGTGTGCATGTATGACGTTTCAGACTTTGTAGAAAAGGACACAGACTTGCTTGATCCG
      S  F  V  A  T  S  G  N  A  N  R  M  Q  F  G  V  N  H  Y  A  G  V  C  M  Y  D  V  S  D  F  V  E  K  D  T  D  L  L  D  P
1921  GCTTTCGTCCCACTGTTGAGGAATTCCAGTGAAGCTTTGTCGCCAAATTATTCTCTGGCCCAAGTTTGGCAGCGGAGAAGCACTCAAGTCAATCGTTGTCCAAGCACAAGTC
      A  F  V  P  L  L  R  N  S  S  E  S  F  V  A  K  L  F  G  S  P  S  L  A  A  E  K  H  Y  K  D  E  S  I  V  V  Q  A  Q  V
2041  TCCTCCAGACCACTGCGTACCCTCACACCATTCGTTTCCTCCAATTTCACAGGGAGAAGCGAACGTACTTCAGAAGATGCACAAGAACACCTCGAGCTCGACAGAGGTAAATCTTACCCT
      S  S  R  P  L  R  T  L  T  P  F  V  S  S  N  F  T  G  R  S  E  R  T  S  E  D  A  Q  E  H  L  E  L  D  R  G  K  S  Y  P
2161  GTTACCACTCAAATCACTTCACTCTTTCCGAGCTCTTTGCCAACATCTCCAAAGCCCGTCTCTGGGCATTGTCTTGCATTCGTCCGAACGACTCCGGCTCAACAAATTCTTTTGACAAAC
      V  T  T  Q  I  T  S  L  F  P  S  S  L  P  T  S  P  K  P  V  S  G  H  C  L  A  F  V  R  T  T  P  A  H  Q  I  L  L  T  N
2281  GCCGAGtcaaagctcaaattcgtagtctcctcctatctgatttggcctcccgtcgcagTGTCGAATACATCGCCGACTTTGACCTGGCTCAATTCTGTGGCCGTTATGTCCCGACTATGC
      A  D                                                     V  E  Y  I  A  D  F  D  L  A  Q  F  C  G  R  Y  V  P  T  M  R
2401  GTGGGGTCTGAGAGTGAGCGAATCACACAATGTGCTCGAGCGAATGGGTGGATTGAGGGTGTAGATTATGTTGTTGGCCACCGCTCGATATGGCTTTCGTATTCCGCTTGGAAAATGGTAG
      G  S  E  S  E  R  I  T  Q  C  A  R  A  N  G  W  I  E  G  V  D  Y  V  V  G  H  R  S  I  W  L  S  Y  S  A  W  K  M  V  E
2521  AAGATACTAGTTCGGGCTGTAGAAAAGACGCCAAGAGGGCTTTAGGGGACGACGATGGTAGCGATGAGGAGGCACAGTGTCGCACCGGACGATGGCACAGAATACACGCATG
      D  T  V  R  A  V  E  K  D  A  K  R  A  L  G  D  A  G  M  E  M  D  D  D  E  D  A  S  V  A  P  D  D  G  T  E  Y  T  H  E
2641  AAGGAGGAGGTTATGGTTATGGGGTGTTGGATCCCAAATGGACGGTGGGTGGACTCGGAGCGAGTAATGATGACTTGGTATTGAGGAGGACAGGAACAAACGGAACTCAACATCGTTCCC
      G  G  G  Y  G  Y  G  V  L  D  P  K  W  T  V  G  G  L  G  A  S  N  D  L  V  L  R  R  T  G  T  N  G  T  Q  H  R  S  P
2761  CTAATCAAGGTCCAGCATACAATGCTCTACCGGCCCCGAATTCCCCAGCTCAAATGTCGACGCCGAACGCGTTCAGAGGCCAGGCGGATGATGGCGGTTGGGGATCCGAATGGGATAAGA
      N  Q  G  P  A  Y  N  A  L  P  A  P  N  S  P  A  Q  M  S  T  P  N  A  F  R  G  Q  A  D  D  G  G  W  G  S  E  W  D  K  K
2881  AGGATGAGTCGTCAGTTACGGGGACAGGTACAGTCCCTGCTGGGTCATCTGTCAAGGAAGGTGATGGTCTTGTCGTCAAAGACGCTCCAGACTCTGTCGAAGAAGTTCCTTCAACAAGGA
      D  E  S  S  V  T  G  T  V  P  A  G  S  S  V  K  E  G  D  G  L  V  V  K  D  A  P  D  S  V  E  E  V  P  S  T  R  S
3001  GCCGGAGATTCTGGCTTGGAACTGTCTGGGCTTGTACTTGGTTCATTCCCTCGTTTTTGCTGACGCATGTTGGGCGGATGAAACGACCTGATGTTCGACGTGCATGGAGAGAAAAGGTCA
      R  R  F  W  L  G  T  V  W  A  C  T  W  F  I  P  S  F  L  L  T  H  V  G  R  M  K  R  P  D  V  R  L  A  W  R  E  K  V  T
3121  CCATTTGTTTCCTCATTTTGCTTCTCAACGGCATCGTCATTTTCTACATTGTCATTTTTGGCCGGTTACTTGCCCCGACTATGATAATGCATGGTGACGAACGAGGTCGCAGAACATA
      I  C  F  L  I  L  L  L  N  G  I  V  I  F  Y  I  V  I  F  G  R  L  L  C  P  D  Y  D  N  A  W  L  T  N  E  V  A  E  H  T
```

terminal MMD and a C-terminal CSD have been isolated and characterized from some filamentous, dimorphic and polymorphic fungi that produce true hyphae (Aufauvre-Brown et al. 1997; Fujiwara et al. 1997; Park et al. 1999; Zhang et al. 2000; Chigira et al. 2002; Müller et al. 2002; Amnuaykanjanasin and Epstein 2003, 2006; Madrid et al. 2003; Niño-Vega et al. 2004; Liu et al. 2004; Takeshita et al. 2006), and these chitin synthases have been grouped into class V or VI. Comparisons by Clustal W analysis of the deduced amino acid sequence of LeChs1 with those of previously classified class V and class VI chitin synthases (Table 1) suggest the unique character of LeChs1, as mentioned below. When comparisons were performed with overall amino acid sequences and with the C-terminal CSD sequences, LeChs1 displayed higher similarities to the class

VI chitin synthases (29%–46% identity and 60%–68% identity, respectively) than to the class V chitin synthases (26%–29% and 54%–58%, respectively). In contrast, the N-terminal MMD of LeChs1 displayed higher similarity to those of the class V chitin synthases (18%–21%, except for AfChsE) than to those of the class VI chitin synthases (13%–15%). In all three comparisons, the differences between the degrees of similarity of LeChs1 to the class V and those to the class VI chitin synthases were small enough to consider that LeChs1 has a unique intermediary character between class V and VI enzymes. This idea is also supported by the feature of the MMD of LeChs1. The MMDs of the known class V chitin synthases contain the ATP-binding motifs (P-loop, GESGAGKT; switch I; TASKAG; and switch II, DFPGF) (Fujiwara et al. 1997; Liu et al. 2004),

Fig. 1. *Continued*

```
3241 CTGCAGATAATACTACTATGTCGCTATTCAAGGAAAAGTCTACGATGGTCTCAAACTTCGTTCAAGGCCAACACAGTGTATCGACGGAGAGGACTCCAACTCCGATGATGTCTTGGAAG
       A  D  N  T  T  M  S  L  F  K  E  K  S  T  M  V  S  N  F  V  Q  G  Q  H  S  D  I  D  G  E  D  S  N  S  D  D  V  L  E  A

3361 CGCTTGCTGGACTGGATTTGACATATTACTTCCCAGTTCCTCTTGTCCTCGGCTGCGGTAATCTTGGTCCGACCTCAACGATGAAGCTGTCGTTCAAGAACTTCACTGAAACCGAACCGA
       L  A  G  L  D  L  T  Y  Y  F  P  V  P  L  V  L  G  C  G  N  L  G  P  T  S  T  M  K  L  S  F  K  N  F  T  E  T  E  P  T

3481 CTGCCGGATCATTCGTCAGGAGAGTTTGCTCAATCTACGACGAGCCAGCTTCATAATTCGGATTGGTATACTGCGACGTTCCAACCCGTCATCAATCAGTATCACATTGGAACGCTAGTTC
       A  D  H  S  S  G  E  F  A  Q  S  T  T  S  Q  L  H  N  S  D  W  Y  T  A  T  F  Q  P  V  I  N  Q  Y  H  I  G  T  L  V  H

3601 ACAGTTGGGATGAGATACAGTCCTATGCATCAGATGAAGACATTGAAAGtaagtaaatggatatctacattaccaaaaattgacaatatttgttcagGATCTGGGGTGTCTATAACAAC
       S  W  D  E  I  Q  S  Y  A  S  D  E  D  I  E  K                                                     I  W  G  V  Y  N  N

3721 AATCTCTATGATCTGACTGACTACTTCTACACTCAAGACCAAAATGACAACAATGACGCATATCTTTTCCTCGACTCTGGTATCGAGACCGTTTTCAAGGATCGTTCTGGCCAAGGCATC
       N  L  Y  D  L  T  D  Y  F  Y  T  Q  D  Q  N  D  A  Y  L  F  L  D  S  G  I  E  T  V  F  K  D  R  S  G  Q  G  I

3841 ACCGATTCGTTGAATACCGCGCTCGGGAAGCTTAACGAGACCTATCGTCAACAGAATCTGGACTGTCTCAACAATGTCTTCTACATTGGCGAGACTGACTTCCGCACTACCGCCAGGTGC
       T  D  S  L  N  T  A  L  G  K  L  N  E  T  Y  R  Q  Q  N  L  D  C  L  N  N  V  F  Y  I  G  Q  T  D  F  R  T  T  A  R  C

3961 CAAGCGCCAAACATTACGCTGATCGTGGTAGCTGCTATTCTGATGTCTACAATGGTTTTGAAATGTTTGTATCTCTACTTATTTTGTgtgttgttttttcatcagcctctacagttctttc
       Q  A  P  N  I  T  L  I  V  V  A  A  I  L  M  S  T  M  V  L  K  C  L  Y  L  L  F  C

4081 tgctctgcagcttgctcctaaacgtacccggagTTGCAAGACAAATTTGTGCTCTGCCAAGTTCCTTGTTACACAGAAGGGGAAAAATCTCTTCGCCGCACTATTGACTCCCTTGCTGCT
                                          L  Q  D  K  F  V  L  C  Q  V  P  C  Y  T  E  G  E  K  S  L  R  R  T  I  D  S  L  A  A

4201 CTCAACTACGACGACAAGCGCAAACTTATCTTTCTCATCTGCGATGGAAACATTACCGGAAGCGGGAACGACCGTACCACGCCCCATCGTCCAGGATATTCTCGGTGTGGATCCTAAG
       L  N  Y  D  D  K  R  K  L  I  F  L  I  C  D  G  N  I  T  G  S  G  N  D  R  T  T  P  R  I  V  Q  D  I  L  G  V  D  P  K

4321 CTTGACCCTGAACCATTGATGTTCAAGTCGGTCGGAGAAGGCTCACGTGCGCTAAATTATGGCAAAGTGTACTCCGGGTTATATGAGTTCGAAGGACACGTCGTGCCgtatgtccttatg
       L  D  P  E  P  L  M  F  K  S  V  G  E  G  S  R  A  L  N  Y  G  K  V  Y  S  G  L  Y  E  F  E  G  H  V  V  P

4441 atagcttcaggaaaaatttcctgactcttttctcaagGTACATGGTTGTTGTGAAAGTTGGTAAACCCACGGAGCGTTCAAAACCTGGCAATCGTGGCAAGCGTGACAGTCAAATTTTGC
                                             Y  M  V  V  V  K  V  G  K  P  T  E  R  S  K  P  G  N  R  G  K  R  D  S  Q  I  L  L

4561 TTATGCATTATTTAAACCGTGTTCACTTTGACGCGCCTATGAGTCCGCTAGAGCTGGAGCTGTATCATCAGATGAGGAATGTTATTGGGATCGATCCTGCCTTCTACGAGTATATATTCA
       M  H  Y  L  N  R  V  H  F  D  A  P  M  S  P  L  E  L  E  L  Y  H  Q  M  R  N  V  I  G  I  D  P  A  F  Y  E  Y  I  F  T

4681 CCATCGACGCCGACACAgtgagttggtttgtttgttgaaccatatggagttagtgaaactcagttcctgtttgcagACTGTGACACCAGAATCTCTCAACAGGCTTGTAGCTTCGGCCGCTAT
       I  D  A  D  T                                                              T  V  T  P  E  S  L  N  R  L  V  A  S  A  A  I

4801 CGACTCGAATATCATTGGAATATGTGGCGAAACCAAGGTAGCGAACGAGGATACTTCCTGGTGGACAATGATCCAGGTTTACGAGTATTACATATCCACCACTTGACCAAGGCCTTCGA
       D  S  N  I  I  G  I  C  G  E  T  K  V  A  N  E  D  T  S  W  W  T  M  I  Q  V  Y  E  Y  Y  I  S  H  H  L  T  K  A  F  E

4921 AAGTCTCTTCGGCTCCGTCACCTGTCTTCCCGGTTGCTTCAGTCTTTACCGTATTCGTACAGCAGACAAAGGTCGGCCTATCATCATATCGAACCGTGTCATTGACGAGTACGCCGAACC
       S  L  F  G  S  V  T  C  L  P  G  C  F  S  L  Y  R  I  R  T  A  D  K  G  R  P  I  I  I  S  N  R  V  I  D  E  Y  A  E  P

5041 CAACGTTGATACCCTTCACAAGAAGAATCTTTTCTCTTTGGGAGAAGATCGTTATTTGACGACACTGTTGATGAAACACTTTCCTACGTTCAAGACAAATTCAACCCTGATGCTGTAGC
       N  V  D  T  L  H  K  K  N  L  F  S  [L  G  E  D  R  Y  L]  T  T  L  L  M  K  H  F  P  T  F  K  T  K  F  N  P  D  A  V  A

5161 CCATACGGTCGCTCCGGAGTCTACTCGTGTGCTATTCTCTCAACGTCGTCGTTGGATCAACTCCACGGTCCATAACCTGTGTGAATTGGTGATGCTCCCTGAGCTCATCGGATTCTGCTG
       H  T  V  A  P  E  S  T  R  V  L  F  [S  Q  R  R  R  W]  I  N  S  T  V  H  N  L  C  E  L  V  M  L  P  E  L  I  G  F  C  C

5281 GTTTTTCTATGCGTATATTCGTTTTTGTTGTTGATCTTTTGAGTACCATGgtgaatgactgtgccgttctttcattctatatattcgtttgttgacttccatcgtagATCCTGCCTTCCACCg
       F  S  M  R  I  F  V  F  V  F  D  L  L  S  T  M                                                              I  L  P  S  T

5401 tcgtctatgtgtgtgcactcacttcttacttcttattcctgtcctaacctgaaatgtcagCTGGTATACCTTGTTATCGTAGTTTCTCTTGGAAAAATACCGCTTCCCGTTGATTGCCATC
                                                                  L  V  Y  L  V  I  V  V  S  L  G  K  Y  R  F  P  L  I  A  I

5521 ATCATGATTGCAGCTGGGTATGGTCTTCAGgtgagtttaatccattgctgcgttgcgccactcgttaacatcttgacacagGTTTTGATCTTCATTTTGAAGCGTGAATTCATGCTGATC
       I  M  I  A  A  G  Y  G  L  Q                                                     V  L  I  F  I  L  K  R  E  F  M  L  I

5641 GGGTGGATGGTCGTTTACCTTCTTTCGtaagtccttcgataccgaagcagggcttttctcactattttcgcagGTATCCAATCTACAGCTTATTCCTTCCGGTTTATTCCTTCTGGTGCA
       G  W  M  V  V  Y  L  L  S                                               Y  P  I  Y  S  L  F  L  P  V  Y  S  F  W  C  M

5761 TGGACGAATTCGGTTGGGGTAACACTCGTTTGGTTATCGGTGAAGGAAAAGACAAGAAGGTCATTGTAAATGACGATGATAAATTTGACGAGTCGATGATTCCACTGAAGAAGTTTAGTG
       D  E  F  G  W  G  N  T  R  L  V  I  G  E  G  K  D  K  K  V  I  V  N  D  D  D  K  F  D  E  S  M  I  P  L  K  K  F  S  E

5881 gtgcgtgtttccattccatattcatccgcatggtgactgactgcttattgtagAATACGAAGCTGAAGCATGGGAAACTGGTTCGCGTCATTCAGATGAAACTGGATATAGTAAGCCGCA
                                                             Y  E  A  E  A  W  E  T  G  S  R  H  S  D  E  T  G  Y  S  K  P  H

6001 CACACATCCGCGTCCAACCGCATCTCGGACTGGTTCTCCCTACAACTATCAACAAGGCTCACAAGCTGGTGATTACTATCGTGACACGAACTTAACGATGAATAGCCGTTCGAATCCCAA
       T  H  P  R  P  T  A  S  R  T  G  S  P  Y  N  Y  Q  Q  G  S  Q  A  G  D  Y  Y  R  D  T  N  L  T  M  N  S  R  S  N  P  N

6121 TCCATTCACGGGGAGCCAGCAATCTTTAAGCAACCTTTCACATCATGGTGGTCAGCAATACGCTGCACCTCAACTCCCTTACATGCCTTTTGGTGGTGGGCCAGCTCTGCCGCCGGCTCT
       P  F  T  G  S  Q  Q  S  L  S  N  L  S  H  H  G  G  Q  Q  Y  A  A  P  Q  L  P  Y  M  P  F  G  G  G  P  A  L  P  P  A  L

6241 GATTATGGCGGACGGATGCCTATGGGACCAGTTATGCCAATGGGCTATCAGAACACTGGCAgtgtatgtgggatgccgatggggatggggatgaatatgatgtctcctgccgctatggat
       I  M  A  D  G  C  W  D  Q  L  C  Q  W  A  I  R  T  L  A

6361 ccccgaaccactatgatggctggtatgatgactggtggtagctttggtggctctcagTCTGGTGCATTCGCCCCACCTACCATGCCTGCCAGTCAAGAGCAGCGACCGTTGTCCACATTT
                                                                S  G  A  F  A  P  P  T  M  P  A  S  Q  E  Q  R  P  L  S  T  F

6481 TCGATGGCGACCACTGTCAACCCATTTGCTGGTTCCAGCCAGAACCCCAATCCTAGTGATGACGAACTTTTCGCCGCATTGCGCAGGTCTTACCTCGGTACTCAAGACCTGATGACTGTTACC
       S  M  A  T  T  V  N  P  F  A  G  S  S  Q  N  P  N  P  S  D  D  E  L  F  A  A  L  R  S  Y  L  G  T  Q  D  L  M  T  V  T

6601 AAGAAGTACGTGTTATTATGCCCAGTTGATGATGATTTTTTACTCATGTGTTGTAGAACCGCTCGAGGAGCCATGAATGCTAAATTCCCGAGAGCGGACCTACTGTCGCGCAAAGACTTCC
       K  K  Y  V  L  L  C  P  V  D  D  D  F  Y  S  C  V  V  E  P  L  E  E  P  *

6721 TCAACAAGTCCATTGACACTATCCTTGCGGAATAGAATATAGACCCTTTCATTCGTT
                                        _____
                               polyadenylation signals
```

which are considered to be essential for ATPase and motor activities, whereas these motifs are not conserved in the MMDs of the class VI chitin synthases (Chigira et al. 2002; Takeshita et al. 2006). In the MMD of LeChs1, we found P-loop-like (GITGSGKT) and switch II-like (DQPGY) sequences, but the switch I motif was not conserved. LeChs1 displayed the highest degree of identity to UmChs6 (46% overall amino acid identity and 68% identity in the CSDs). UmChs6 from the dimorphic hemibasidiomycete *Ustilago maydis* (DC.) Corda, which has been classified into class VI chitin synthase (Chigira et al. 2002), does not contain an MMD (Garcerá-Teruel et al. 2004). A phylogenetic tree of LeChs1 and the other class V and VI chitin synthases listed in Table 1 demonstrated that LeChs1 could be classified into the class VI chitin synthases (Fig. 2). The phylogenetic tree showed that LeChs1 and UmChs6, both of which are from basidiomycetes, were represented as a new subgroup within the class VI chitin synthase cluster. This subgroup was distant from the subgroup of the other four class VI

chitin synthases, all of which are from ascomycetes. This finding may reflect the evolutionary distance between basidiomycetes and ascomycetes.

LeChs1 contains two highly conserved domains, LGE-DRYL and SQRRRW, which are implicated as essential catalytic sites of chitin synthases (Nagahashi et al. 1995; Yabe et al. 1998) (see Fig. 1). Hydrophobicity analysis by the SOSUI program (http://sosui.proteome.bio.tuat.ac.jp/ sosui_submit.html) suggested that LeChs1 contains eight putative transmembrane domains in the CSD and between the MMD and the CSD. This result is consistent with the fact that chitin synthases are known to be membrane-bound proteins. The schematic representation of the structural features of LeChs1 is shown in Fig. 3.

Before the present study, the physiological functions of the class V and VI chitin synthases and their MMDs had been investigated extensively on CsmA (class V) and CsmB (class VI) from *Aspergillus nidulans* (Eidam) Vuill. (Horiu-chi et al. 1999; Takeshita et al. 2005, 2006). Takeshita et al.

Table 1. Comparison of the deduced amino acid sequence of LeChs1 with those of other class V and VI chitin synthases

Class	Gene product	Accession no.	Percent (%) identity to LeChs1		
			Full length (1–1937)	MMD (19–621)	CSD (1228–1752)
VI	LeChs1	AB262360	100	100	100
	UmChs6	AF030554	46	–	68
	AnCsmB	AB230981	30	15	60
	AoChsZ	AB081655	30	14	61
	CgChsA	AY064208	30	14	62
	PbChs4	AF107624	29	13	61
V	AfChsE	Y09542	29	11	55
	AoChsY	AB081656	28	20	58
	WdChs5	AF469116	28	21	55
	AnCsmA	AB000125	28	18	57
	FoChsV	AF484941	27	18	54
	BgChs2	AF189366	27	18	57
	MgCsm1	AB018251	26	20	54

The values of percent (%) identity to LeChs1 were calculated by the ClustalW program. The myosin motor-like domain (MMD) and the chitin synthase domain (CSD) of each chitin synthase were predicted on the basis of the results of InterProScan analysis: UmChs6 (full length: 1–1103, MMD: – , CSD: 383–916), AnCsmB (1–1739, 22–600, 1013–1541), AoChsZ (1–1760, 19–694, 1036–1564), CgChsA (1–1783, 19–613, 1060–1568), PbChs4 (1–1774, 19–704, 1044–1572), AfChsE (1–1498, 1–413, 854–1384), AoChsY (1–1857, 18–776, 1218–1748), WdChs5 (1–1885, 15–774, 1214–1745), AnCsmA (1–1852, 18–775, 1214–1744), FoChsV (1–1863, 20–766, 1212–1745), BgChs2 (1–1867, 13–758, 1203–1734), MgCsm1 (1–1869, 19–766, 1200–1730)
Um, *Ustilago maydis*; Af, *Aspergillus fumigatus* Fresen; An, *Aspergillus nidulans*; Ao, *Aspergillus oryzae* (Ahlb.) E. Cohn; Bg, *Blumeria graminis* (DC.) Speer; Cg, *Colletotrichum graminicola* (Ces.) G.W. Wilson; Fo, *Fusarium oxysporum* Schltdle; Mg, *Magnaporthe grisea* (T.T. Hebert) M.E. Barr; Wd, *Wangiella dermatitidis* McGinnis; Pb, *Paracoccidioides brasiliensis* (Splend.) F.P. Almedia
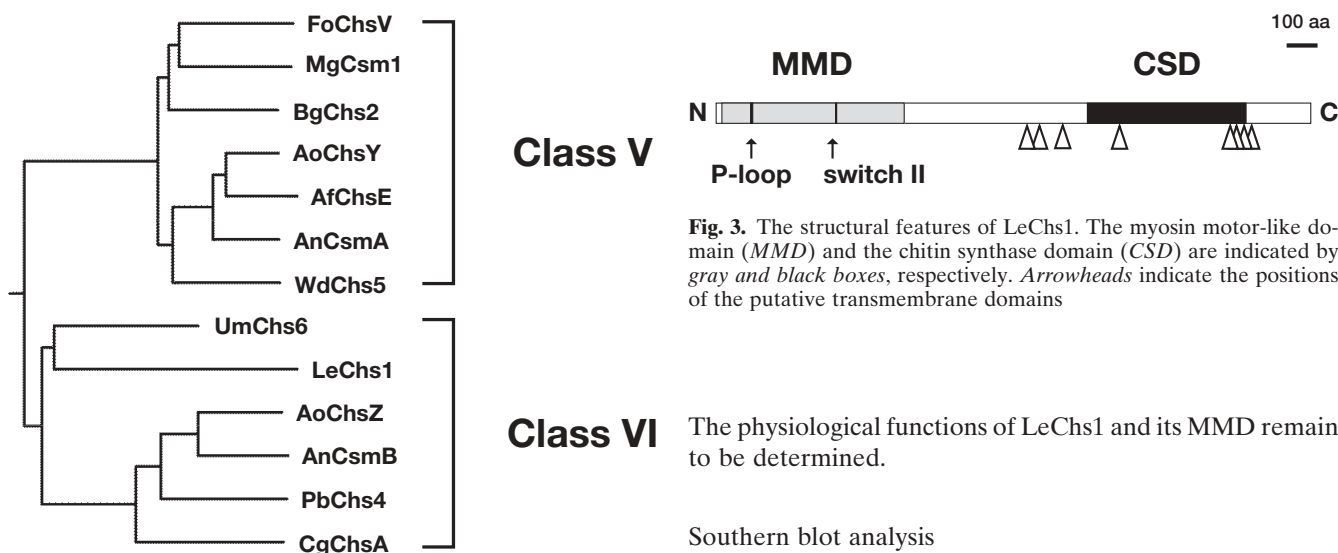
Fig. 3. The structural features of LeChs1. The myosin motor-like domain (*MMD*) and the chitin synthase domain (*CSD*) are indicated by *gray and black boxes*, respectively. *Arrowheads* indicate the positions of the putative transmembrane domains

Fig. 2. Phylogenetic tree of class V and VI chitin synthases. The tree was constructed using the ClustalW program. Um, *Ustilago maydis*; Af, *Aspergillus fumigatus* Fresen; An, *Aspergillus nidulans*; Ao, *Aspergillus oryzae* (Ahlb.) E. Cohn; Bg, *Blumeria graminis* (DC.) Speer; Cg, *Colletotrichum graminicola* (Ces.) G.W. Wilson; Fo, *Fusarium oxysporum* Schltdle; Mg, *Magnaporthe grisea* (T.T. Hebert) M.E. Barr; Wd, *Wangiella dermatitidis* McGinnis; Pb, *Paracoccidioides brasiliensis* (Splend.) F.P. Almedia

(2005, 2006) reported that CsmA is involved in polarized chitin synthesis in a manner dependent on the interaction between the MMD and actin cytoskeleton and that CsmA and CsmB perform compensatory functions that are essential for hyphal tip growth of *A. nidulans*. The authors also suggested that the MMDs of CsmA and CsmB may function as anchors of CsmA and CsmB to maintain localization near actin structures at hyphal tips and in forming septa.

The physiological functions of LeChs1 and its MMD remain to be determined.

Southern blot analysis

To assess the copy number of *Lechs1* in the *L. edodes* genome, Southern blot analysis was conducted using the 1070-bp DNA fragment of *Lechs1* (nucleotides 4098 to 5167 in Fig. 1) as a probe. The *L. edodes* genomic DNA digested with *Eco*RI, *Pvu*II, or *Sal*I was analyzed. As shown in Fig. 4, a single hybridization signal was detected in all three digests. The commercially cultivated *L. edodes* strain (dikaryon) used in this study is assumed to have few allelic differences in the genome, as already mentioned; therefore, it is conceivable that few different restriction fragment length polymorphisms attributed to the alleles are found between haploid genomes in the distinct nuclei of the cell. From this point of view, the result obtained by the Southern blot analysis may indicate that the *L. edodes* strain contains *Lechs1* as a single-copy gene per haploid nuclear genome.

Fig. 4. Southern blot analysis of *Lentinula edodes* genomic DNA. Genomic DNA digested with *Eco*RI (*lane 1*), *Pvu*II (*lane 2*), or *Sal*I (*lane 3*) was size-fractioned by 0.8% agarose-gel electrophoresis and transferred onto a nylon membrane. The Southern blot was hybridized with a 1070-bp *Lechs1* probe
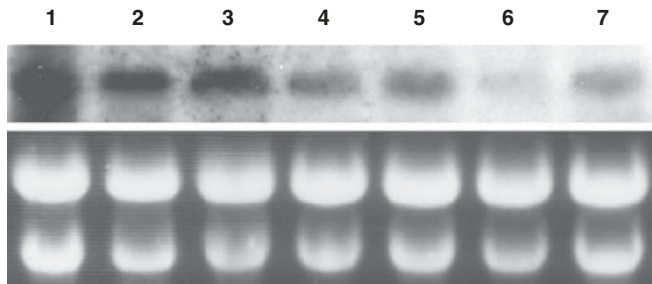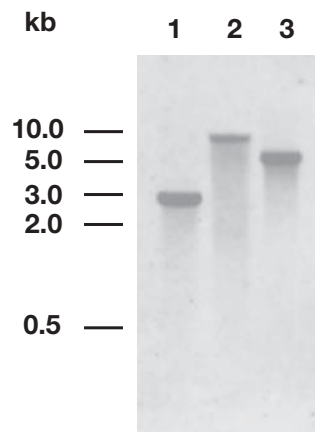

Fig. 5. Northern blot analysis of *Lechs1* expression in the course of fruit-body formation of *L. edodes*. Total RNA samples (30 μg each) isolated from vegetative mycelia grown on potato dextrose agar (PDA) (*lane 1*), pileus and stipe of fruit bodies at maturation stage I (*lanes 2 and 3*, respectively), those at maturation stage II (*lanes 4 and 5*, respectively), and those at maturation stage III (*lanes 6 and 7*, respectively) were analyzed using the same probe that we applied to the Southern blot analysis (see Fig. 4). *Upper panel*, Northern blot; *lower panel*, loading control to ensure the equal loading of RNA samples based on rRNA levels in the ethidium bromide-stained gel

Expression analysis of *Lechs1*

We examined the transcriptional expression patterns of *Lechs1* in the course of fruit-body formation and in the different tissues of the fruit bodies (stipe and pileus) of *L. edodes* by Northern blot analysis. Total cellular RNA was isolated from vegetative mycelia grown on PDA medium and fruit bodies at the different stages of maturation (stages I–III, from early to late). Each fruit body was separated into stipe and pileus before RNA isolation. The RNA samples were subjected to analysis using the same probe that we applied to the Southern blot analysis described above (Fig. 4). The ethidium bromide-stained ribosomal RNA (rRNA) bands are shown as a loading control in Fig. 5. As shown in Fig. 5, a single hybridization signal was detected in all RNA blots, but the signal intensities were different among the RNA blots. The high transcriptional levels were observed in vegetative mycelia (lane 1) and in the fruit body (both stipe and pileus: lanes 2 and 3, respectively) at maturation stage I (immature fruit body). The transcriptional levels in both stipe and pileus of the fruit bodies gradually declined as fruit-body maturation progressed (lanes 4–7). These results indicated that *Lechs1* is constitutively transcribed dur-

ing the fruit-body formation of *L. edodes*, but its transcription is regulated temporally in a fruit body-maturation-dependent manner. The stipe contained a slightly higher level of transcript than the pileus when compared at the same stage of fruit-body maturation. The *Lechs1* gene is transcribed more actively in the vegetative mycelia and the fruit body at the early stage of maturation (stage I) than in the fruit bodies at the middle and late stages of maturation (stages II and III, respectively), suggesting that LeChs1 may function more actively at the vegetative mycelial stage as well as at the beginning stage of fruit-body development of *L. edodes*. However, it should be noted that we did not confirm that the transcriptional levels of *Lechs1* in vegetative mycelia grown on PDA medium and sawdust medium are the same.

In contrast to our results, Sreenivasaprasad et al. (2000) reported that the transcriptional level of the class III chitin synthase gene of *A. bisporus* (a basidiomycetous mushroom) was very low at the mycelial stage and high in the fruit bodies, and that no detectable difference in the transcriptional levels was observed among fruit bodies at the different maturation stages. Our results combined with those of Sreenivasaprasad et al. (2000) suggested that chitin synthases belonging to different classes have different functions in the fruit-body development of the basidiomycetous mushrooms and that their genes are differently regulated.

In conclusion, we have, for the first time, identified and characterized the *L. edodes* gene (*Lechs1*) encoding a class VI chitin synthase with an MMD, which may be of value in investigating the morphogenesis (fruit-body development) of *L. edodes* and other basidiomycetous mushrooms. Studies aimed at clarifying the physiological functions of LeChs1 as well as identifying chitin synthases belonging to other classes in *L. edodes* are in progress.

References

Amnuaykanjanasin A, Epstein L (2003) A class V chitin synthase gene, *chsA* is essential for conidial and hyphal wall strength in the fungus *Colletotrichum graminicola* (*Glomerella graminicola*). Fungal Genet Biol 38:272–285

Amnuaykanjanasin A, Epstein L (2006) A class Vb chitin synthase in *Colletotrichum graminicola* is localized in the growing tips of multiple cell types, in nascent septa, and during septum conversion to an end wall after hyphal breakage. Protoplasma 227:155–164

Aufauvre-Brown A, Mellado E, Gow NAR, Holden DW (1997) *Aspergillus fumigatus chsE*: a gene related to *CHS3* of *Saccharomyces cerevisiae* and important for hyphal growth and conidiophore development but not pathogenicity. Fungal Genet Biol 21:141–152

Bertani G (1951) Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 62:293–300

Bowen AR, Chen-Wu JL, Momany M, Young R, Szaniszlo PJ, Robbins PW (1992) Classification of fungal chitin synthases. Proc Natl Acad Sci USA 89:519–523

Bulawa CE (1993) Genetics and molecular biology of chitin synthesis in fungi. Annu Rev Microbiol 47:505–534

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

Cid VJ, Durán A, Rey FD, Snyder MP, Nomblea C, Sánchez M (1995) Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. Microbiol Rev 59:345–386

Doedt T, Krishnamurthy S, Bockmühl DP, Tebarth B, Stempel C, Russell CL, Brown AJP, Ernst JF (2004) APSES proteins regulate morphogenesis and metabolism in *Candida albicans*. Mol Biol Cell 15:3167–3180

Dutton JR, Johns S, Miller BL (1997) StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans*. EMBO J 18:5710–5721

Fujiwara M, Horiuchi H, Ohta A, Takagi M (1997) A novel fungal gene encoding chitin synthase with a myosin motor-like domain. Biochem Biophys Res Commun 236:75–78

Garcerá-Teruel A, Xoconostle-Cázares B, Rosas-Quijano R, Ortiz L, León-Ramírez C, Specht CA, Sentandreu R, Ruiz-Herrera J (2004) Loss of virulence in *Ustilago maydis* by *Umchs6* gene disruption. Res Microbiol 155:87–97

Horiuchi H, Fujiwara M, Yamashita S, Ohta A, Takagi M (1999) Proliferation of intrahyphal hyphae caused by disruption of *csmA*, which encodes a class V chitin synthase with a myosin motor-like domain in *Aspergillus nidulans*. J Bacteriol 181:3721–3729

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 148:1335–1347

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

Kües U, Liu Y (2000) Fruiting body production in basidiomycetes. Appl Microbiol Biotechnol 54:141–152

Lerner MR, Boyle JA, Mount SM, Wolin SL, Steitz JA (1980) Are snRNPs involved in splicing? Nature (Lond) 283:220–224

Litzka O, Papagiannopolous P, Davis MA, Hynes MJ, Brakhage AA (1998) The penicillin regulator PENR1 of *Aspergillus nidulans* is a HAP-like transcriptional complex. Eur J Biochem 251:758–767

Liu H, Kauffman S, Becker JM, Szaniszlo PJ (2004) *Wangiella (Exophiala) dermatitidis* WdChs5p, a class V chitin synthase, is essential for sustained cell growth at temperature of infection. Eukaryot Cell 3:40–51

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

Madrid MP, Pietro AD, Roncero MIG (2003) Class V chitin synthase determines pathogenesis in the vascular wilt fungus *Fusarium oxysporum* and mediates resistance to plant defence compounds. Mol Microbiol 47:257–266

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 148:4025–4033

Munro CA, Gow NA (2001) Chitin synthesis in human pathogenic fungi. Med Mycol 39:41–53

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

Park IC, Horiuchi H, Hwang CW, Yeh WH, Ohta A, Ryu JC, Takagi M (1999) Isolation of *csm1* encoding a class V chitin synthase with a myosin motor-like domain from the rice blast fungus, *Pyricularia oryzae*. FEMS Microbiol Lett 181:131–139

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

Sassoon J, Mooibroek H (2001) A system of categorizing enzyme-cell wall associations in *Agaricus bisporus*, using operational criteria. Appl Microbiol Biotechnol 56:613–622

Sheppard DC, Doedt T, Chiang LY, Kim HS, Chen D, Nierman WC, Filler SG (2005) The *Aspergillus fumigatus* StuA protein governs the up-regulation of a discrete transcriptional program during the acquisition of developmental competence. Mol Biol Cell 16:5866–5879

Smits GJ, Kapteyn JC, Ende Hvd, Klis FM (1999) Cell wall dynamics in yeast. Curr Opin Microbiol 2:348–352

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

Steidl S, Papagiannopoulos P, Litzka O, Andrianopoulos A, Davis MA, Brakhage AA, Hynes MJ (1999) AnCF, the CCAAT binding complex of *Aspergillus nidulans*, contains products of the *hapB*, *hapC*, and *hapE* genes and is required for activation by the pathway-specific regulatory gene *amdR*. Mol Cell Biol 19:99–106

Takeshita N, Ohta A, Horiuchi H (2002) *csmA*, 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 59:1380–1394

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

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

Wu J, Miller BL (1997) *Aspergillus* asexual reproduction and sexual reproduction are differentially affected by transcriptional and translational mechanisms regulating *stunted* gene expression. Mol Cell Biol 17:6191–6201

Yabe T, Yamada-Okabe T, Nakajima T, Sudoh M, Arisawa M, Yamada-Okabe H (1998) Mutational analysis of chitin synthase 2 of *Saccharomyces cerevisiae*: identification of additional amino acid residues involved in its catalytic activity. Eur J Biochem 258:941–947

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene (Amst) 33:103–19

Zhang Z, Hall A, Perfect E, Gurr SJ (2000) Differential expression of two *Blumeria graminis* chitin synthase genes. Plant Pathol 1:125–138