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Cloning and Phylogenetic Analysis of Chitin Synthase Genes from *Tricholoma matsutake*

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Chitin synthases (UDP-N-acetyl-D-glucosamine: chitin 4-β-N-acetyl-D-glucosaminyl transferase, EC 2.4.1.16) catalyze the synthesis of chitin from UDP-N-acetyl-D-glucosamine. Two zymogenic type of chitin synthase gene (*TmCHS1* and *TmCHS2*) were amplified and its nucleotide sequences were determined. By the amino acid comparison and UPGMA tree grouping, TmChs1 and TmChs2 were classified as class II and class IV chitin synthases respectively. The class II type TmChs1 was grouped with others of Agaricales ectomycorrhizal mushroom. Additionally the phylogenetic tree was well adapted to Hymenomycete previously classified by morphological and physiological characteristics.

KEYWORDS: *Tricholoma matsutake*, **Chitin synthase**

Chitin, the β-1,4-linked polymer of N-acetylglucosamine, is an important structural component of fungal cell walls and it is one of the components that has been used as a taxomic marker (Bartnick-Garcia *et al.*, 1978). Chitin is not found in plant or mammals and control of chitin synthesis must be an important aspect of fungal growth (Cohen, 1987). Therefore chitin synthases (UDP-N-acetyl-D-glucosamine :chitin 4-β-N-acetyl-D-glucosaminyl transferase, EC 2.4.1.16) that catalyze the synthesis of chitin from UDP-N-acetyl-D-glucosamine deserve attention in that they could be studied for development of antifungal drugs with high selectivity and low toxicity as well as for understanding of the molecular mechanism of hyphal growth and differentiation.

There are three types of chitin synthases, chitin synthase 1, 2, and 3 in *Saccharomyces cerevisiae*, and they are encoded by the three different genes designated by chitin synthase gene 1 (*CHS1*), 2 (*CHS2*) and 3 (*CHS3*), respectively (Bulawa *et al.*, 1992; Silverman *et al.*, 1989; Valdivieso *et al.*, 1991). Shaw *et al.* and Cabib *et al.* have revealed that Chs1 repairs damaged chitin during cell separation, Chs2 is required for primary septum formation, and Chs3 is involved in all other chitin synthesis.

Analyses of DNA fragments from taxonomically diverse fungal species have shown that most fungi have three to six chitin synthases. The *CHS* gene fragments which have significant homology to the *CHS1* and *CHS2* genes of *S. cerevisiae* were grouped into three classes (I, II, III) by Bowen *et al.* (1992). Bowen *et al.* (1992) designated degenerated PCR primers by using two amino acid sequences homologous among three different chitin synthase gene products of yeasts (*S. crervisiae* Chs1, and Chs2, and *C. albicans* Chs1). They classified chitin synthase into three classes, I, II, and III, by comparing their deduced amino acid sequences, and supposed that this classification reflects functional difference of chitin synthases. The information obtained from *CHS* gene analysis has been used in phylogenetic and taxonomic studies of fungi (Bowen *et al.*, 1992). In addition, The Mehmann *et al.* (1994) were classified the chitin synthases of ectomycorrhizal fungi into four categories, class I, II, III and IV, following the conserved gap region.

Many ascomycete and basidiomycete genera form ectomycorrhial symbioses with gymnosperm and angiosperm trees. The mutualistic association enhances the ability of the host plant to obtain nutrients and water from the soil, and the plant provides the fungus with its primary source of carbohydrates (Harley, 1989).

Tricholoma matsutake (S. Ito et Imai Singer), its Korean name Song-yi, is an ectomycorrhizal fungus which belongs to Trhcholomataceae, Agaricales, Hymenomycetes, and Basidiomycotina (Brunett *et al.*, 1976; Hawksworth *et al.*, 1983).

Presently the chitin synthase were not studied in *Tricholoma* sp. Therefore, evaluation of the chitin synthase as a taxomonic marker and applicability to phylogentic study is required in the genus *Tricholoma*. In this paper, we report the conserved sequence region of two cloned chitin synthase gene from *T. matsutake* and classify the cloned chitin synthase by comparing to other chitin synthase. In order to understand the phylogenetic position of *T. matsutake* as a representative species of the genus *Tricholoma*, the maximum parsimony analysis with other fungal chitin synthase is presented.

Materials and Methods

Mushroom and genomic DNA preparation. Fruiting

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bodies of *Tricholoma matsutake* were harvested at Gachang near in Daegu, Korea. Immediately after harvesting, it was extensively washed with sterilized water to remove soil and other contaminants and then it was frozen at -70°C for storage. After lyophilization for two days, fruiting bodies were used in this study. *T. matsutake* genomic DNA was extracted according to the method as described by Kim *et al.* (1990) with some modifications: the 250 mg of lyophilized fruiting bodies were ground to a fine powder for 10 min in liquid nitrogen in a precooled mortar and pestle at 4°C. Twenty milliliters of ice-cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris-HCl, pH 7.4) were added and stirred for 10 min. SDS was added to a final concentration of 2% (w/v) and incubated for 1 hour at 55°C. Centrifugation was carried out at 10,000 g for 10 min and the supernatant was decanted. NaCl was added to a final concentration of 1.4 M and followed by adding one-tenth volume of 10% CTAB buffer (10% CTAB, 100 mM EDTA, 500 mM Tris-HCl, pH 8.0). After mixing throughly and incubating for 10 min at 65°C, chloroform : isoamylalcohol (24 : 1, v/v) extraction was repeated until the interface layer was clear. Nucleic acids were concentrated by precipitation in a two volumes of absolute ethanol and dissolved in 0.1× TE buffer (0.1 mM EDTA, 1 mM Tris-HCl, pH 8.0). RNase A was added to remove RNA. A purity of the final DNA solution was measured and yielded OD260/OD280 values of 1.7 to 1.8.

PCR amplification and analysis of nucleotide sequence. The chitin synthase gene fragments were amplified by two primers. Following the Bowen *et al.* (1992) the forward primer was derived from the position of 450-458 amino acid sequence (5'-CTGAGCTTACTATGTATAATGAGGAT-3') and reverse primer was from the position of 650-658 amino acid sequence (5'-GTTCTGGAGTTTGTATTCGAAGTTCTG-3') in Chs1 of *S. serevisiae*. PCR was done in 500 μl microcentrifuge tube containing 0.2 μM each of dNTP, 0.2 M each of primers, 1.5 mM $MgCl_2$ and 0.5 units of Taq DNA polymerase in amplification buffer (5 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.01 mM EDTA, 0.1 mM DTT, 5% glycerol and 0.1% Triton X-100). Cycling parameters were programmed as follows: prewarm 2 min at 95°C, 30 sec at 95°C, 30 sec at 50°C, and 1 min at 72°C for 30 cycles.

pGEM T-Easy vectors (Promega Co. Madison, USA) were used to clone the PCR-amplified DNA fragments. The determined nucleotide sequence was analyzed with DNASTAR program and the BLAST program used to search the Genbank and EMBL database.

The classification of chitin synthase and phylogenetic analysis. For the determination of cloned chitin synthase class, we analyzed the exon-intron junction following other chitin synthase gene fragment. The deduced amino acid sequences were aligned with other ectomycorrhizal fungi and determined the gap region. For the comparison with other ectomycorrhizal fungi, we used the species previously determined by Mehmann *et al.* (1994). Chitin synthase genes from various organism were downloaded from EMBL, GenBank (Table 1). The collected sequences were

Table 1. Taxonomic affinities of fungal species and chitin synthase-encoding gene designations used in this study

Class	Order	Family	Species	Gene designation	Accession nos.
Ascomycota	Schizosaccharomycetales	Schizosaccharomycetaceae	*Schizosaccharomyces pombe*	*SpCHS1*	Z54308(EMBL)
	Elaphomycetales	Elaphomycetacea	*Elaphomyces muricatus*	*ElmCHS1*	X78091(EMBL)
				ElmCHS2	X78092(EMBL)
	Tuberales	Tuberacea	*Tuber uncinatum*	*TuuCHS1*	X78101(EMBL)
Basidiomycota	Agaricales	Cortinariaceae	*Helbeloma crustuliniforme*	*HecCHS1*	X78093(EMBL)
			Helbeloma mesophaeum	*HemCHS1*	X78094(EMBL)
			Cortinarius odorifer	*CooCHS1*	X78089(EMBL)
				CooCHS2	X78090(EMBL)
		Tricholomatacea	*Laccaria laccata*	*LalCHS1*	X78095(EMBL)
				LalCHS2	X78096(EMBL)
			Tricholoma matsutake	*TmCHS1*	AY056586(GenBank)
				TmCHS2	AY056586(GenBank)
	Boletales	Boletaceae	*Boletus edulis*	*BoeCHS1*	X78087(EMBL)
			Xerocomus badius	*XebCHS1*	X78102(EMBL)
	Hymenogastrales	Rhizopogonaceae	*Rhizopogon vulgaris*	*RhvCHS1*	X78098(EMBL)
				RhvCHS2	X78099(EMBL)
	Russulales	Russulaceae	*Russula adulterina*	*RuaCHS1*	X78100(EMBL)
			Lactaritus deterrrimus	*LcdCHS1*	X78097(EMBL)
	Appyllophorales	Cantharellaceae	*Cantharellus cibarius*	*CacCHS1*	X78088(EMBL)
	Ustilaginales	Ustilaginaceae	*Ustilago maydis*	*UmCHS1*	X87748(EMBL)
				UmCHS2	X87749(EMBL)

aligned with chitin synthase gene of *T. matsutake* with the use of the computer program CLUSTAL W (Thompson *et al.*, 1994) under the UNIX environment. After alignment with amino acid sequences of other chitin synthase, the sets of sequences were conformed to the format of PHYLIP package (version 3.5) (Felsenstein, 1993). For the UPGMA analysis, we used PRODIST program and NEIGHBOR program. In addition, to analyze the phylogenetic relationship among other species, PAUP* program was used to obtain the most parsimonious trees (Swofford, 1998).

Results and Discussion

Sequence analysis of *TmCHS1* and *TmCHS2*. In order to chracterize chitin synthase genes of *T. matsutake*, we cloned two different chitin synthase genes and designated as *TmCHS1* and *TmCHS2* respectively. The cloned fragment of *TmCHS1* and *TmCHS2* were sequenced and submitted to Genbank database (*TmCHS1*; GenBank AY056586, *TmCHS2*; GenBank AY056587). By the comparison of conserved amino acid and analysis of acceptor/donor (AG/GT) consensus sequence, the deduced amino acid sequence was proposed (Fig. 1). Therefore it was presumed that *TmCHS1* had one intron sequence (202-252 nt.) and *TmCHS2* had six intron sequences (30-76 nt., 219-263 nt., 312-363 nt., 542-586 nt., 650-696 nt., 737-792 nt.). Additionally we supposed that these intron region could be used as a DNA marker for detection of species in *T. matsutake*. Then the two amino acid sequences were aligned to other chitin synthase sequences of ectomycorrhizal mushroom for chitin synthase type classification and phylogenetic analysis (Fig. 2).

Classification of chitin synthase. Mehmann *et al.* indicated that the chitin synthase has four zymogenic types. In chitin synthase zymogenic type classification the conserved amono acid gap region and UPGMA grouping was introduced (Bowen *et al.*, 1992; Mehmann *et al.*, 1994). The putative amino acid of *TmCHS1* and *TmCHS2* were classified following these methods. As shown in Fig. 2, the *T. matsutake* TmChs1 was class II type chitin synthase and TmChs2 was class IV type. In the aligned amono acid sequence, the TmChs2 had five gap regions

TmChs1 189 amino acid sequence

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  1 EELFCRTMHGVMKNVAHLCKRDRSKTWGKDGWKKVVICIVSDGRAKVNSRTLSVIAAMGA
 61 YQEGVAKTKIGDAPVTAHIYEYTTQISVTPSMKIEGPERGTVPVQLIFCLKEKNQKKINS
121 HRWFFNAFGPILQPNVCVLLDVGTMPGPTSIYHLWKAFDINSNVGGACGEIVALKGKWGL
181 NLLNPLVAA
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TmChs2 187 amino acid sequence

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  1 RSLFTKTMHNVIKNIAHLCSRNKSKTWGPDGWKKVVVCIVSDGRSKVNKRTLHVLSVMGC
 61 FQEGIPKAQVAGKTLTCHALRYTSNVVVSESGEVSQGSCPVQIVFCLKEQNKKKLNSHRW
121 FFNAFGPLIKPNVCILLDVGTKPTGTSIYELWKCFDKHSYVGGACGEICVDTGRGCSLLL
181 TSPLAAS
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Fig. 1. Deduced amino acid sequence from *TmCHS1* (GenBank accession AY056586) and *TmCHS2* (GenBank accession AY056587) gene. The intron sequence in the *TmCHS1* and *TmCHS2* was excised by consensus acceptor/donor sequence.

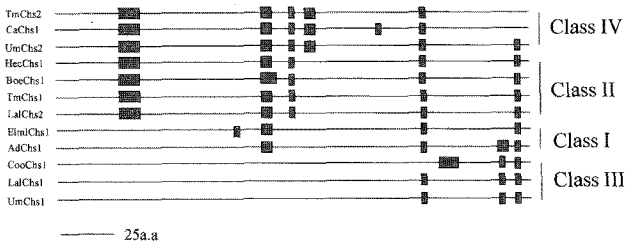

Fig. 2. The schematic diagram that indicate gap region of chitin synthase conserved domain. The four classes of chtin synthase zymogenic type was represented as class I, II, III and IV. The gap region was indicated by blacken box. The amino acid sequence of conserved doamin region was aligned and employed to gap region analysis.

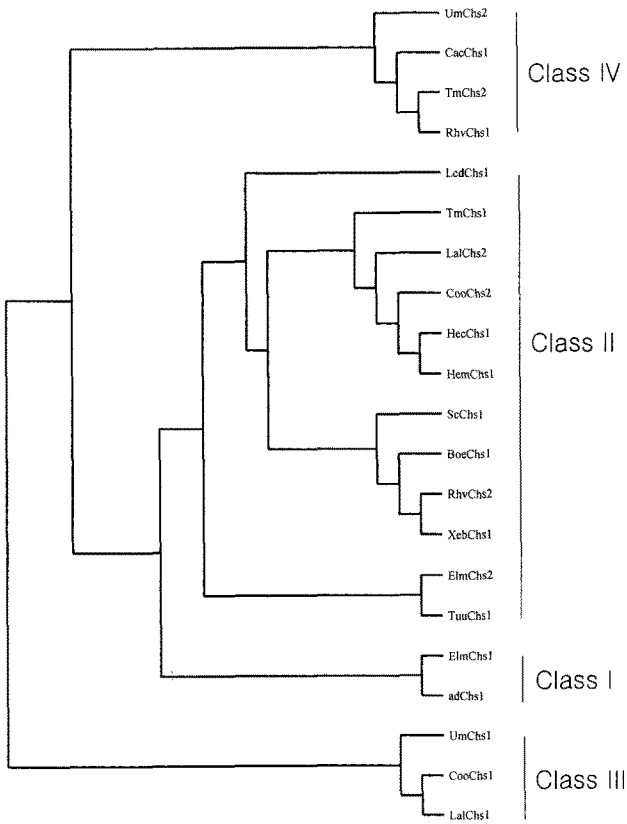

Fig. 3. The UPGMA tree was constructed using PAM matrix of PRODIST program in phylip package (3.5c). The four classes of chitin synthase were grouped.

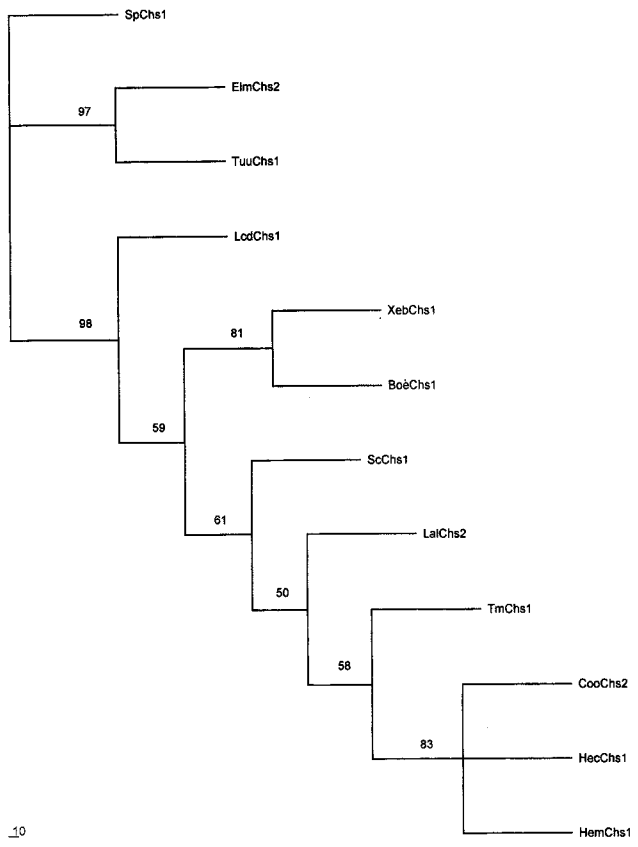

Fig. 4. The maximum parsimony tree resulted from putative amino acid of 12 class II chitin synthase. The bootstrapping value were indicated as the numbers above branch.

applicable to class IV chitin synthase and also TmChs1 had five gap regions, but those were differently located, applicable to class II chitin synthase. When the class IV chitin synthase was compared to class II chitin synthase, the class IV chitin synthase had another gap region, and it was shown that these two type chitin synthase were differently derived. The UPGMA tree also indicated clearly four types of zymogenic chitin synthase (Fig. 3). In addition the most abundant chitin synthase type is class II as previously described by others.

Phylogenetic application. To define the phylogenetic relationship of *T. matsutake*, we used putative amino acid from *TmCHS1*, which was falled within class II chitin synthase as viewed in Fig. 3. Because the class II chitin synthase was abundantly existed we adopted the TmChs1 sequence and compared to other class II chitin synthase from previously determined ectomycorrhizal species in the research of Mehmann *et al.* We used the maximum parsimony algorithm with heuristic search option. The 100 bootstrap replicate were introduced to abtain confidence value with TBR swapping algorithm, COLLAPSE and MULTREES option. The 50% majority rule consensus tree was represented (Fig. 4). The consistence index was 0.858 and retention index was 0.729. From the resulting maximum parsimony tree the Agaricales, Boletales and Russulales species were likely classified to previously suggested Hymemomycetes classification (Hibbett *et al.*, 1997). Also, the Ascomycota species were clustered and used as outgroup. Althouth the genus level classification was not tested in this study, the chitin synthase amino acid sequence were well adapted to the order level classification.

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