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Isolation and Characterization of a Chitin Synthase Gene Fragments from *Pleurotus sajor-caju*

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여름느타리의 Chitin synthase 유전자 단편분리 및 발현 특성 분석

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ABSTRACT: We isolated three amplified DNA fragments from *P. sajor-caju* by Polmerase chain reaction (PCR) using the chithin synthase specific primers. Since the sequence analysis of the these fragments showed significant homology to the other known chitin synthase gene, we regarded these cloned fragments as *PsCHS1*, *PsCHS2*, and *PsCHS3* according to their size. The *PsCHS3*, which showed the highest sequence homology (83% identity in amino acid level with *ChsI* of *Rhizopus oligosporus* in conserved region), was selected to see expression pattern of the corresponding gene. The result of RT-PCR using internal primer of the *PsCHS3* fragment revealed that *PsCHS3* gene was only expressed in cap and mycelium but not in stipe. In order to see whether the *PsCHS3* gene was to be induced by wounding, the comparison of the mRNA level of this gene between wounded and unwounded mature cap showed at least two times induction of this gene by wounding treatment.

KEYWORDS: Chitin synthase, Organ specific induction, *Pleurotus sajor-caju*

Chitin, a β(1,4)-linked polymer of N-acetylglucosamine (GlcNAc), is a fibrous cellulose-like polysaccharide that serves as a major component of the cell wall of most filamentous fungi (Bowen *et al.*, 1992). The polymer is synthesized by incorporation of GlcNAc unit from uridine diphosphate (UDP)-liked GlcNAc in the reaction catalyzed by chitin synthase. Chitin synthase mainly located in the cell plasma membrane (Gooday, 1977). Biochemical studies have shown that there are two types of enzymes (Cabib and Farkas, 1971): a zymogen form that is activated *in vitro* by proteolytic treatment, and a non-zymogen type which does not require proteolytic activation (Orlean 1987). By aligning the deduced amino acid sequences from 32 partial PCR-amplified CHS homologues showed that each homologue fell into three distinct classes (I, II, III) which probably reflect the functional difference and 14 taxonomically diverse fungal species have at least 1 chitin synthase homologue (Bowen *et al.*, 1992). Thereafter, this strategy has been used to clone from a variety of filamentous fungi. In *Aspergillus nidulans*, four chitin synthase genes, *chs*A, *chs*B, *chs*C, and *chs*D, have been cloned and chracterized (Yanai *et al.*, 1994; Motoyama *et al.*, 1994; Motoyama *et al.*, 1996). In addition, three genes, *CHS*1, *CHS*2 and *CAL*1, from the yeast *Saccharomyces cervisiae*, three from *Candida albicans* (Bulawa *et al.*, 1986; Silveman, 1988; Cabib *et al.*, 1989, Au-Young and Robbins, 1990; Chen-Wu *et al.*, 1992), and three from *Neurospora crassa* have been isolated and charac-

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terized (Yarden and Yanofsky, 1991). The fungal genus Pleurotus that contain a number of edible species is one of the most important edible mushroom. Over recent years, more information has become available from Pleurotus on genes involved in basal metabolism. However, there is no report on the detection of the gene for chitin synthase. In this paper we report the existence of seven different genes related to chitin synthase in *Pleurotus sajor-caju* and showed the expression pattern of one of them for its relationship with defense mechanism and organ specificity.

Materials and Methods

Strain and growth media

P. sajor-caju (ASI 2070) obtained from National Institute of Agricultural Science and Technology was used in this experiment. This strain was maintained on mushroom complete medium (MCM; 0.5g $MgSO_4 \cdot 7H_2O$, 0.46g KH_2PO_4, 1.0g K_2HPO_4, 2.0g Peptone, 2.0g Yeast extract, 20g Glucose) at 30°C. pBluescriptIIKS(+) and *Escherichia coli* DH 5α strain were used as a cloning vector and host cell, respectively. *E. coli* was grown in Luria Bertani (LB) medium at 37°C for propagation of plasmid.

Preparation of genomic DNA and RNA from mushroom

Mycelia of *P. sajor-caju* were harvested by filtration through a Whatman NO. 1 filter paper and washed with deionized water. Washed mycelia were frozen in liquid N2 and powdered in a mortor. Genomic DNA was isolated from the mycelia using a rapid extraction procedure described by Graham (1994). Total RNA was extracted from cap, stipe and mycelium by a modified lithium chloride (LiCl) precipitation protocol according to Sambrook *et al.* (1989).

cDNA library construction and phage DNA isolation

Poly(A)$^+$ RNA was purified from total RNA using a poly(A) Quik mRNA isolation Kit (Stratagene). cDNA library was constructed using a ZapII, cDNA synthesis Kit (Stratagene) and a GigapackII Kit (Stratagene). Lambda phage DNA was isolated from cDNA library by Sambrook *et al.* (1989).

Polymerase chain reaction

Amplification of *CHS* gene fragments from *P. sajor-caju* genomic DNA and cDNA library was performed using two oligo deoxynucleotide primer sets which were specially designed by sequence analysis of the conserved regions of the previously reported *CHS* genes. P1 (5'-TGGGGATCCCARGTNTAYGARTAYTA-3') and P2 (5'-ATAGAATTCTTAATCCAICKICKICKYTG-3') were used as one of the CHS specific primer set and P3 (5'-CARAAYTTYGARTAYAAR-3') and P4 (5'-ATAGAATTCTTIAICCAICKICKIC KYTG-3') were used as the other set, in which R denotes A or G, Y denotes C or T, I denotes inosine, and K denotes G or T. PCR was performed in a total volum of 100 μL containing 10 X Taq polymerase buffer (Promega), 2 mM $MgCl_2$, 200 μM dNTP, 1 μM of each primer, 80 ng of template DNA, and 1.5 unit of Taq polymerase (Promega). Amplification was performed in a thermal cycler (Perkin Elmer Cetus) for 40 cycles with each cycle of 30 sec at 94°C, 30 sec at 48°C and 1 min at 72°C. The PCR products were eletrophoresed on a 0.8% agarose gel and visualzed by UV transilluminator after staining with ethidium bromide (EtBr).

Cloning, Sequencing and Homology analysis

The PCR products were separated on 1.4% agarose gels, and the bands of the desired size were excised and eluted using a QIAquick Gel Extraction Kit (QIAGEN). Purified

PCR products were subcloned into the pBluescriptIIKS(+) digested with EcoRV restriction enzyme. Transformation was carried out using *E. coli* strain DH5α. Plasmid DNA from transformants was isolated according to standard procedures (Sambrook *et al.* 1989). Insert DNAs in plasmids were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using a sequenase version 2.0 kit (USB) according to the manufacturer's instructions. DNA homology analysis was carried out with Dnasis program (HITACHI) and Genbank database (NIH). Amino acid homology analysis was performed with Prosis program (HITACHI) and Swiss-plot database.

Wound treatment

The cap of mature fruitbody was cut by razor blade from the edge to inside about 5cm in length. After 4 hours, tissue adjascent to the mechanically wounded site was collected and immediately frozen in liquid N_2. Total RNA was extracted from the wounded and unwounded cap tissues at similar developmental stage for RT-PCR analysis.

Reverse Transcription (RT)-PCR

Reverse transcription was carried out in a 80 μL reaction mixture containing 5 X buffer (Promega), 0.25 mM each of the 4 deoxynucleotides (dNTPs), 2 μg of synthetic poly dT primer (5'-CGAGGAATTCTTTTTTTTTTT-TTTTTTTTTTTTTTTTTTTT-3'), 200 unit of Moloney Murine Leukaemia Virus (M-MLV) Reverse Transcriptase, and 8 ug DNAse treated RNA which had been pre-heated at 72°C for 2 min. The reactions were performed at 37°C for 60 min and 10 min at 72°C for enzyme inactivation. The 15 μL of first strand cDNA products was then employed as template DNAs for the amplification of *PsCHS3* cDNA using PCR in a 100 μL volume, containing 10 X buffer (Promega), 10 m*l* of 25 mM $MgCl_2$, 0.25 mM each dNTP, 0.5 μg of *PsCHS3* specific primer (5'-CCTTGAGAGTG-TGTTCGG-3'), and 2.5 units of Taq. DNA polymerase. PCR was performed by using a DNA thermal cycler (PerKin Elmer Cetus) for one cycle of 5 min at 94°C, then 40 cycles of 30 sec at 94°C, 30 sec at 48°C and 2 min at 72°C. The RT-PCR products were resolved by electrophoresis on 1.4% agarose gels, and then stained with EtBr for photography.

Results and Discussion

Isolation of chitin synthase (*CHS*) gene fragments by PCR

The oligonucleotide primer sets for isolating *CHS* gene fragments were designed based on analysis of highly conserved regions derived from previously reported chitin synthase proteins such as *Aspergillus fumigatus* ChsE, *A. nidulans* ChsE and ChsA, and *Candida albicans* Chs2. These primer sets, P1 and P2 (set A), P3 and P4 (set B) were used for PCR amplification of *CHS* gene fragments from *P. sajor-caju* genomic DNA and cDNA library. PCR using the primer set A generated one band of 350bp from both genomic DNA and a cDNA library. In PCR using the other primer set B, two bands, 600bp (clear one) and 500bp (faint one), were produced from a cDNA library (Fig. 1). These three PCR amplified DNA fragments were purified from the agarose gel using a QIAQUIK gel extraction kit and subcloned into the pBluescriptIIKS(+) for nucleotide sequencing analysis.

Nucleotide sequence analysis and comparison of deduced amino acid sequences

After subcloning the PCR products into pBS+(KS), partial nucleotide sequencing of the three clones (designated *PsCHS1*, *PsCHS 2*, and *PsCHS3*) were done by the dideoxy method (Sanger *et al.*, 1977). The partial nucleotide sequence and the deduced amino acid sequence of the PCR amplified DNA

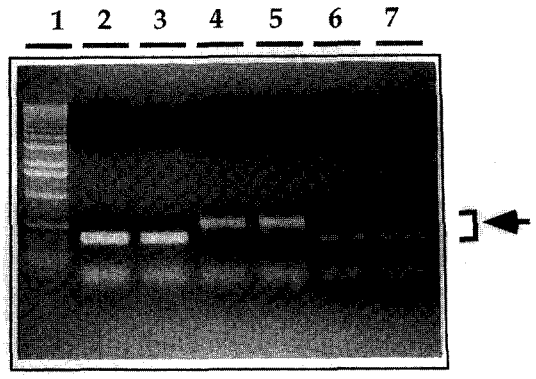

Fig. 1. Isolation of genomic and cDNA fragments produced by PCR with Chitin synthase specific primers. Total genomic DNA (lane 2 and 3) and cDNA (lane 4, 5, 6, and 7) of *Pleurotus sajor-caju* were isolated and then used in PCR reaction with different sets of Chitin synthase specific primers, set A (2, 3, 6, and 7) and set B (4 and 5), to isolate Chitin synthase genes. The PCR products were separated by electrophoresis on an 1.4% agarose gel with 1 kb ladder DNA size marker (1). Arrow indicates position of the PCR products.

PsCHS1
CGAGCTTGTAGCCAAAGCTGGAGACCGATGGACACTCACCTATGTCAAACC
E L V A K A G D R W T L T Y V K P
CAGTAAGGCCGAGACTGATGTTCCCGAGTCCGCTGCGGAATTGATTGGTCA
S K A E T D V P E S A A E L I G Q
GAGCGGCAGCTGGTTCAAGAATTCTATAAGCCGAATTCTCGCAGATATCCA
S G S W F K N S I S R I L A D I H
TCACAC
H T

PsCHS2
TTTGAGTACAAGATCAGCAATATATTGGACAAGACGACGGAGCATGTTTGG
F E Y K I S N I L D K T T E H V W
CTACATCAGCGCGCTGGTGATGGACGAACTATGCTCCAGTCCCTAAAGTTG
L H Q R A G D G R T M L Q S L K L
ACCACGGTGCCA
T T V P

PsCHS3
CAGAACTTTGAGTATAAGATGTCCAACATTCTCGATAAACCCCTTGAGAGT
Q N F E Y K M S N I L D K P L E S
GTGTTCGGTTATATCAGTGTTCTGCCGGGTCGATTCAGCGCCTCGATCAAA
V F G Y I S V L P G R F S A Y I K
GGCCTTGCTCAACGGCCCAACGGAAGGGCCGCTACG
G L A Q R P N G R A A T

Fig. 2. Nucleotide and deduced amino acid sequences of the three PCR amplified DNA fragments.

fragments were shown in Fig. 2. Comparison of partial nucleotide sequence of the each fragment with the known fungale *CHS* sequences showed 64~82% matches (Data is not shown). This result suggested that these three cDNA fragments are putative *CHS* gene fragments and there might be at least three *CHS* genes in *P. sajor-caju*. Homology analysis of amino acid sequence in conserved region showed that these three *PsCHS1, PsCHS2,* and *PsCHS3* genes have 81.6%, 81.3%, and 83% identity with *CHS1* of *A. nidulans*, *CHS2* of *C. albicans*, and 83% of *R. oligosporus*, respectively (Table 1). By the investigation of Bowen *et al.* (1992), *CHS* genes from 14 fungal species are divided into three distinct classes according to their sequence homology,

Table 1. Similarity of deduced amino acid sequences of *Pleurotus sajor-caju CHS* gene fragments with homologus sequences from other fungi

P. sajor-caju gene	Best matches			Class	Species
	Gene	Identity (%)	Similarity (%)		
PsCHS1	AdCHS1	81.6	97.1	I	*Aspergillus nidulans*
	NcCHS1	74.8	97.1	I	*Neuropsora crassa*
	NcCHS1	64.6	97.1	II	*N. crass*
PsCHS2	CaCHS2	81.3	87.5	I	*Candida albicans*
	CHS1	81.3	87.5	I	*Saccharomyces*
	CHS2	81.3	87.5	II	*S. cerevisiae*
PsHCS3	RoCHS2	83	88.1	II	*Rhizopus oligosporus*
	RoCHS1	78.6	90.5	II	*R. oligosporus*
	AdCHS2	78	85.7	II	*A. nidulans*

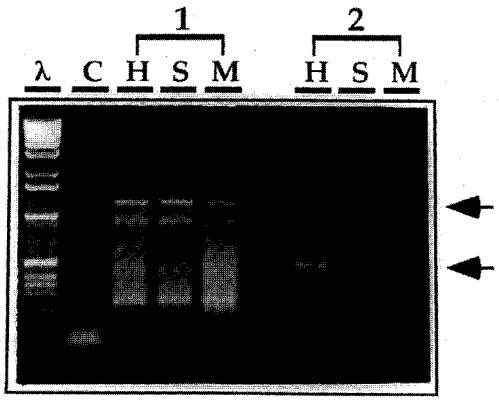

Fig. 3. The *PsCHS3* gene expressed in cap and mycelium tissue but not in stipe. Total RNA (2 ug) from cap (H), stipe (S), and mycelium tissue (M) were subjected to RT-PCR reaction to analyze the *PsCHS3* gene expression pattern in various stages or organs (2). RNA from each treatment was used in cDNA synthesis using Chs specific antisense primer and then amplified by PCR with the redesigned sense primer, corresponding to nucleotide sequence of the isolated cDNA fragment of the *PsCHS3* gene, and oligo dT primer as an antisense primer. The RT-products were separated by electrophoresis on an 1.4% agarose gel with 1 kb ladder DNA size marker (l). The result of control reaction using water instead of RNA and same primers is shown in Lane C. Same amount of RNA from each treatment was also subjected to RT-PCR reaction using 18S rRNA specific primers as a control (1). Arrow indicate position of the RT-PCR products generated by 18S rRNA specific primers (upper one) and the *PsCHS3* gene specific primers (lower one).

CHS1, CHS2, and CHS3, meaning that they are separate functional groups. Based on this analysis, it seems that *P. sajor-caju PsCHS1* and *PsCHS2* belong to CHS1 group and the *PsCHS3* belong to CHS2 group.

Organ specific expression of the *PsCHS3* gene

To characterize the function of *CHS* genes in *P. sajor-caju*, the expression pattern of *PsCHS3*, was analyzed by RT-PCR method. First, organ specificity of this gene expression was investigated by comparison of mRNA level of this gene in different organs including cap, stipe, and mycelium, using newly designed *PsCHS3* specific primer (see materials and methods). The result of RT-PCR experiment revealed that this gene was expressed in cap and mycelium but not in stipe (Fig. 3). This gene showed relatively high expression in cap, while it showed only slight expression in mycelium, suggesting that the products of *CHS* genes might perform different fuctions with different localizations or have different roles during development in *P. sajor-caju*. In *S. cerevisiae*, indeed, three chitin synthase gene have been cloned so far; each one has a different fuction. Recent evidences indicated that *CHS1* gene acts as a repair enzyme during cell separation (Cabib *et al.*, 1989, 1992). *CHS2* gene is responsible for chitin formation in the central disk of primary septum (Silverman *et al.*, 1988; Shaw *et al.*, 1991). Chitin synthase III is required for the formation of the chitin ring at the base of the bud and laterial wall during vegetative growth (Shaw *et al.*, 1991, Valdivieso *et al.*, 1991).

Wound induction of the *PsCHS3* gene

In order to study the possibility that CHS is involved in defense mechanism, wound induction of the *PsCHS3* gene was analyzed by comparison of mRNA level of this gene between wounded and unwounded mature cap tissue (see Materials and Methods). The result of this experiment analyzed using densitometer showed that mRNA level of the *PsCHS3* gene was slightly increased at least two times by wound treatment (Fig. 4). It is suggested that this gene product might be related to self defense system by the way of rapid repair of the damaged cell wall. However, additional molecular biological and biochemical experiments for the *CHS* genes shoud be followed to elucidate the function of

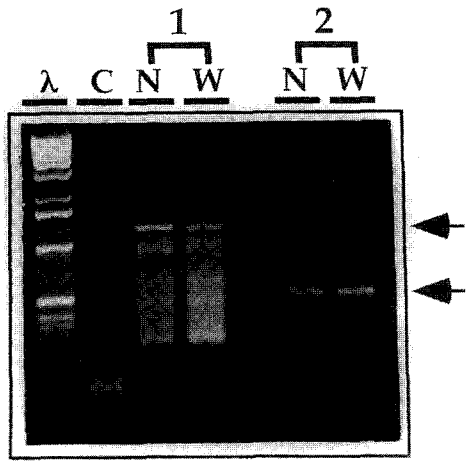

Fig. 4. The expression of the *PsCHS3* gene is induced by wound treatment. Total RNA (2 ug) from wounded (W) and unwounded (N) cap tissue were subjected to RT-PCR reaction to analyze the *PsCHS3* gene induction by wounding (2). RNAs from each treatment were used in RT-PCR reaction as same as explained in Figure 3. The RT-PCR products were separated by electrophoresis on an 1.4% agarose gel with 1 kb ladder DNA size marker. The result of control reaction using water instead of RNA or 18S rRNA specific primers are shown in (C) and (1), respectively. Arrows indicate position of the RT-PCR products generated by 18S rRNA specific primers (upper one) and the *PsCHS3* gene specific primers (lower one).

CHS in *P. sajor-caju*.

적 요

여름 느타리 *Pleurotus sajor-caju*로부터 Chitin synthase(*CHS*) gene 특이 primer를 이용한 PCR을 통해 3개의 DNA 단편을 분리하여 cloning 하였다. 분리된 DNA 단편들을 기존에 보고된 *CHS* 유전자들과의 염기서열을 분석한 결과, 이들 DNA 단편들 3개가 모두 *CHS* 유전자의 단편임을 확인하였고, 또한 이들은 각각 서로 다른 종류의 *CHS* 유전자들임을 알 수 있었다. 한편, RT-PCR 방법을 이용하여 분리된 유전자의 발현 실험을 실시해본 결과, 이들중 하나인 *PsCHS3* 유전자는 갓과 균사에서만 발현되는 기관특이 발현 특성을 보였으며, 또한 이 유전자는 상처 처리에 의해 그 발현이 증가되는 것을 확인하였다. 이러한 실험결과로 볼 때 *P. sajor-caju*의 경우, 다른 균류들의 경우처럼 다양한 기능을 가진 여러 종류(최소 3종류)의 *CHS* 유전자를 보유하고 있으며, 이들 각각은 다른 기관, 또는 다른 생육 단계에 작용하고 있을 것으로 생각되고, 특히 병 방어 기작에도 관여할 것으로 추측되어진다.

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