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Cloning and Phylogenetic Analysis of Chitin Synthase Gene from Entomopathogenic Fungus, *Beauveria brongniartii*

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DNA fragments homologous to chitin synthase gene were amplified from the genomic DNA of *Beauveria brongniartii* by PCR using degenerate primers. Cloning and sequencing of the PCR-amplified fragments led to the identification of a gene, designated *BbCHS1*. Comparison of the deduced amino acid sequence of *BbCHS1* with those of other Euascomycetes revealed that *BbCHS1* is a gene for class II chitin synthase. The BlastP search of the deduced amino acid sequence of *BbCHS1* displayed the highest rate of similarity, 95.8%, with *CHS2* of *Metarhizium anisopliae*. Phylogenetic analysis of the amino acid sequences confirmed the taxonomic and evolutionary position of *B. brongniartii*, which was previously derived by traditional fungal classification based on morphological features.

Key words: *Beauveria brongniartii*, chitin synthase gene, PCR, phylogeny

The use of entomopathogenic fungi for the biological control of many plant-injurious insects has been drawing an increasing attention (1, 7, 8, 10). Among those fungi used as mycoinsecticide, the genus *Beauveria* is widely distributed in nature and has a wide spectrum of infectivity toward a wide variety of insects: *Beauveria bassiana* causes disease in more than 700 insect hosts, many pests as well as in economically important insects such as silkworms and honeybees (7, 8). While, *B. brongniartii* causes disease in cockchafers and white grubs (1).

Chitin synthase (CHS), which catalyzes the synthesis of a β-1,4 linked polymer of N-acetylglucosamine using UDP-N-acetylglucosamine as a substrate, performs several essential functions in fungal morphogenesis including vegetative growth, fruit-body formation, and sporogenesis (4). The genetic and molecular studies in yeast *Saccharomyces cerevisiae* suggest that the three kinds of chitin synthase have a specific function in relation to vegetative growth and ascospore formation (3, 20, 23). It has also been known that chitin synthase acts as a count-balancing agent toward the action of chitinase, whose activities have been implicated as the pathogenetic determinant, involved in host invasion (5).

Since the report of Bowen *et al.* (2), who cloned partial DNA fragments of chitin synthase genes (*CHS*s) from 14 fungal species using degenerate PCR primers complementary to the conserved region of amino acid sequences of *S. cerevisiae CHS1, CHS2* and *Candida albicans CHS1*, this strategy has been used to study various aspect of CHS homologues from a variety of fungi (12, 14, 15, 16, 17, 22). The informations obtained from CHS gene analyses have also been used in phylogenetic and taxonomic studies of several fungi (2, 12, 14).

Despite the importance of *Beauveria* spp. as a potential mycoinsecticide, there has been no report on CHS which performs critical roles in vegetative growth and differentiation. Identification, isolation, and characterization of the genes for chitin synthases, therefore, should facilitate the molecular dissection of pathogenicity whose specificity shows strain diversity as well as the production of conidia which are the desired product due to their greater virulence and greater resistance to adverse environmental conditions. In this paper we report the existence of gene for chitin synthase in *B. brongniartii* and describe the phylogenetic relationship between *B. brongniartii* and ascomycetous fungi through the comparison of the deduced amino acid sequence data reported previously.

Materials and Methods

Strain and culture

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B. brongniartii (KCTC6299) was obtained from Korean Collection for Type Cultures and maintained on SDAY (dextrose 10 g, peptone 2.5 g, yeast extract 5 g, agar 20 g per liter) at 25°C.

Isolation of genomic DNA

For DNA isolation, approximately 10^6 spores/ml of spore suspension prepared with 0.05% Tween 80 were inoculated in SDAY broth and shaken for 40 h at 25°C. Mycelia were filtered on Whatman No.1 filter paper under vacuum and lyophilized at -70°C. Genomic DNA was isolated by the method of Hwang *et al.* (9).

Polymerase chain reaction

The degenerate primers complementary to the conserved regions of CHS described previously (2) were synthesized on a model 392 DNA/RNA synthesizer (Applied Biosystems Inc.). PCR was performed in a 100 µl reaction mixture containing 50 pmole of each primer, 300 ng of genomic DNA, 10 µl of 10× PCR reaction buffer (Promega), 100 µM dNTP (USB), 5 units of Taq polymerase (Promega), and D.W. After overlaying mineral oil (Sigma), the reaction mixtures were preheated at 94°C for 2 min, thereafter, 35 amplification cycles were carried out. Each cycle consisted of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C. Finally, an additional 10 min of extension reaction was performed at 72°C for complete extension. Amplification was performed in a PTC-100™ programmable thermal controller (MJ Research Inc.). The PCR products were electrophoresed on a 1.0% agarose gel, stained with ethidium bromide and visualized under UV light.

Cloning and sequencing

The direct cloning strategy of PCR products described previously was used (13). The PCR products separated on agarose gels were excised and cleaned with QIAquick Gel Extraction Kit (Qiagen). Purified PCR products whose termini were filled with the Klenow fragment were blunt-end ligated into a *Eco*RV-cut pBluescript SK+ vector (Stratagene) in the presence of *Eco*RV to prevent self-ligation. Transformation was carried out using *E. coli* strain XL1-blue (18). Plasmid DNAs from transformants were isolated using QIAprep Spin Plasmid Miniprep Kit (Qiagen). Nucleotide sequences of the inserts were determined by dideoxy chain termination (19) using T7 Sequencing™ Kit (Pharmacia Biotech).

Computer analysis

The nucleotide sequence of PCR clone was analyzed through NCBI data search using BlastN to verify which fragment was encoding a gene for CHS. The amino acid from the nucleotide sequence was deduced through Generunner program. The deduced amino acid sequence of the *BbCHS1* was compared with those from Euascomycetes using CLUSTAL W (21). And then a phylogenetic tree was constructed with the method of unweighted pair-group arithmetic average (UPGMA) of the software package MEGA version 1.0 (11). Bootstrap analysis for evolutionary relationship was performed using heuristic search of 1000 replications. For further analysis, a phylogenetic tree was also constructed using FITCH program in Joseph Felsenstein's PHYLIP 3.5c (6).

Nucleotide sequence accession number

The nucleotide sequence data was registered in the GenBank Nucleotide Sequence Database with accession number U93264.

Results and Discussion

PCR cloning and nucleotide sequence analysis

Agarose gel analysis of PCR products revealed

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  1 GAAATTTGCTTCACACGTACCATGCACGCTGTCATGAAGAATATTTCACACTTTTGCTCC
    E  I  C  F  T  R  T  M  H  A  V  M  K  N  I  S  H  F  C  S
 61 CGATCTCGTTCGCGAACTTGGGGCGAGACTGGCTGGCACAAGATTGTCGTCTGCATTATT
    R  S  R  S  R  T  W  G  E  T  G  W  H  K  I  V  V  C  F  I
121 TGCGATGGTCGCCCCAAAATCCACCCCCGTACACTCGATGCCCTTGCTGCAATGGGTGTC
    C  D  G  R  P  K  I  H  P  R  T  L  D  A  L  A  A  M  G  V
181 TTCCAACACGGTATCGCCAAGAACTTTGTCAACAACCGCGCCGTCCAAGCTCATGTTTAC
    F  Q  H  G  I  A  K  N  F  V  N  N  R  A  V  Q  A  H  V  Y
241 GAGTACACCACTCAGGTGTCTCTTGACTCGGATCTCAAATTCAAGGGTGCTGAGAAGGGC
    E  Y  T  T  Q  V  S  L  D  S  D  L  K  F  K  G  A  E  K  G
301 ATTGTCCCTTGCCAGATGATTTTCTGTCTCAAGGAAAAGAACCGTGGCAAGCTCAACTCT
    I  V  P  C  Q  M  I  F  C  L  K  E  K  N  R  G  K  L  N  S
361 CACAGATGGTTCTTCAACGCTTTTGGCCGGGCACTTAACCCCAATATCTGCATCTTGCTC
    H  R  W  F  F  N  A  F  G  R  A  L  N  P  N  I  C  I  L  L
421 GACGTTGGTACGCCTGGCGGGAAATCCCTCTACCACCTTTGGAAGGCCTTTGATACCGAC
    D  V  G  T  P  G  G  K  S  L  Y  H  L  W  K  A  F  D  T  D
481 TCTAACGTGGCCGGCGCATGTGGTGAGATCAAGGCCATGAAGGGCAAGTACGGCTCTAGT
    S  N  V  A  G  A  C  G  E  I  K  A  M  K  G  K  Y  G  S  S
541 CTCCTCAACCCCCTTGTTGCTTCT
    L  L  N  P  L  V  A  S
```

Fig. 1. The nucleotide sequence and the deduced amino acid sequence of the chitin synthase gene fragment of *BbCHS1*. The conserved sequences encoded by the primers are not included.

the presence of three distinct bands, one of 600 bp and the others of much fainter 500 bp and 350 bp. After cloning the PCR products, partial nucleotide sequencing and BlastN search of the inserts were performed. Out of 16 inserts examined, only one sequence from 600 bp PCR products was identified as a gene for CHS and designated *BbCHS1*. This result, however, can not rule out the existence of additional genes for CHS, because most of the fungi, whose CHS gene has been investigated, has shown the existence of more than two *CHS*s in one species. Complete sequencing of *BbCHS1* on both strands revealed that *BbCHS1* contained uninterrupted open reading frames of 564 bp, when the nucleotide sequences of degenerate PCR primers were excluded (Fig. 1).

Table 1. Similarity of deduced amino acid sequence of *B. brongniartii* CHS gene fragment with homologous sequences from other fungi

B. brongniartii gene	Best match[a]			Class
	Gene[b]	Identity (%)	Similarity (%)	
BbCHS1	*MaCHS2*	90.0%	95.8%	II
	PcCHS3	87.3%	93.7%	II
	NcCHS2	86.8%	93.1%	II
	MgCHS1	85.2%	93.7%	II

[a] Similarity was determined using the BLASTP program to search the non-redundant PDB+SwissProt+SPupdate+PIR+GenPept+GPupdate database through GenBank.
[b] Abbreviations: Ma, *Metarhizium anisopliae*; Mg, *Magnaporthe grisea*; Nc, *Neurospora crassa*; Pc, *Penicillium chrysogenum*.

Analysis of deduced amino acid sequences

To identify which CHS the deduced amino acid sequences of *BbCHS1* were most closely related to, the deduced amino acid sequence of *BbCHS1* was compared with the corresponding region of fungal CHSs registered in GenBank database using the BLASTP program (Table 1). Interestingly, the deduced amino acid sequence of *BbCHS1* displayed the highest rate of identity (90.0%) and similarity (95.8%) with that of *MaCHS2* from *Metarhizium anisopliae*, one of the well known entomopa-

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MgCHS1    EYDFTRTMHAVMKNISHFCRRSKSRTWGENGWQRVVVCIVSDGREKIHPRTLDALAAMGVYQHGIAKNYV  70
NcCHS2    EFGFTRTMHAVMKNISHFCSRNKSRTWGADGWQKIVVCVVSDGREIIHPRTLDALAAMGVYQHGIAKNFV  70
PcCHS3    EVGFTRTMHAVMKNISHFCSRNKSRIWGADGWQKIVVCIISDGREKINARTLDALAAMGVYQHGIAKNYV  70
MaCHS2    EICFTRTTHAVMKNISHFCSRSRSRTWGENGWQKIVVCIISDGREKIHPRTLDALAAMGVYQHGIAKNYV  70
BbCHS1    EICFTRTMHAVMKNISHFCSRSRSRTWGETGWHKIVVCIICDGRPKIHPRTLDALAAMGVFQHGIAKNFV  70
          *  **** ********** * .** **  **...***...***  *. **********.*******.*

MgCHS1    NQKAVQAHVYEYTTQVSLDADLKFKGAEKGIVPCQMLFCLKERNQRKLNSHRWFFNAFGKALNPSVCILL  140
NcCHS2    NQKAVQAHVYEYTTQVSLDSDLMFKGAEKGIVPCQMIFCLKEKNQKKLNSHRWFFNAFGKALNPNVCILL  140
PcCHS3    NNRAVQAHVYEYTTQVSLDSDLKFKGAEKGIVPCQMIFCLKEKNQRKLNSHRWFFNAFGRSLNPNVCILL  140
MaCHS2    NNRAVQAHVYEYTTQVSLDADLKFKGAEKGIVPCQLIFCLKEKNSRKLNSHRWFFNAFGKALNPNVCILL  140
BbCHS1    NNRAVQAHVYEYTTQVSLDSDLKFKGAEKGIVPCQMIFCLKEKNRGKLNSHRWFFNAFGRALNPNICILL  140
          *..**********.** **********..*****.*  **********..*** .****

MgCHS1    DVGTRPGGNSLYHLWKAFDTDSNVAGACGEIKAMKGRLGQNLLNPLVAS  189
NcCHS2    DVGTRPGGTSLYHLWKAFDTDSNVAGACGEIKAMKGRFGGNLLNPLVAS  189
PcCHS3    DVGTRPTSNSLYHLWKAFDTDSNVAGACGEIKAMKGRLGSNLLNPLVAS  189
MaCHS2    DVGTRPGSNSLYHLWKAFDTDSNVAGACGEIKAMKGRFGSNLLNPLVAS  189
BbCHS1    DVGT-PGGKSLYHLWKAFDTDSNVAGACGEIKAMKGKYGSSLLNPLVAS  188
          **** *  **************************. * ********
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Fig. 2. Multialignment of the deduced amino acid sequences of 5 fungal chitin synthase-encoding gene fragments. The alignment was derived by CLUSTAL W. Bb: *Beauveria brongniartii*; Ma: *Metarhizium anisopliae*; Mg: *Magnaporthe grisea*; Nc: *Neurospora crassa*; Pc: *Penicillium chrysogenum*. Asterisks indicate complete identities; Dots indicate conservative substitutions.

thogenic fungi. This result is consistent with the traditional classification in which both genus *Beauveria and Metarhizium* are clustered into the same group of mitosporic Ascomycota. Interestingly, the deduced amino acid sequences of the other genes which displayed higher rate of similarity with *BbCHS1* were those from Ascomycota or Fungi Imperfecti. For instance, both *Neurospora crassa* and *Magnaporthe grisea* are members of the Pyrenomycetes of Ascomycota. And *Penicillium chrysogenum* is a member of Fungi Imperfecti, which shows higher taxonomic affinity to Ascomycota (16). This result may reflect some evolutionary relationships between genus *Beauveria* and ascomycetous fungi. This analysis also permitted the classification *BbCHS1*; *BbCHS1* belongs to class II, because all the *CHS*s shown in Table 1 belong to class II *CHS* defined by Bowen *et al.* (2). Multialignment of the deduced amino acid sequence of *BbCHS1* with those of class II *CHS*s from Euascomycetes and *M. anisopliae* confirmed that *BbCHS1* belongs to class II (data not shown). However, it is of interest that the *BbCHS1* encodes one less amino acids than other class II *CHS*s which encode 189 amino acids; The amino acid arginine or lysine at the amino acid position 145 of class II *CHS* was lacking in *BbCHS1* (Fig. 2). The biological meaning of this unique feature of *BbCHS1* may require further investigation.

Phylogenetic analysis

For the taxonomic and phylogenetic study, bootstrap analysis of the deduced amino acid sequence of the *BbCHS1* was carried out with those of the class II *CHS* fragments of ascomyceteous fungi registered in GenBank by the UPGMA method in MEGA program. Also, to gain a more detailed picture of possible phylogenetic relationships and evolutionary distances, a phylogentic tree was constructed using FITCH program in PHYLIP. As shown in Fig. 3 and 4, except *ScCHS2* of *S. cerevisiae* which had been included as an outgroup to root the tree, the class II *CHS*s analyzed were clearly separated into four clusters. These results were well consistent with the traditional classification based on morphological features; cluster one of those belonging to Chaetothyriales (C), cluster two to the Onygenales of Plectomycetes (PO), cluster three to the Eurotiales of Plectomycetes (PE), and cluster four was heterogenous including both *B. brongniartii* and *M. anisopliae*. *M. grisea* and *N. crassa* clustered into the same group with *B. brongniartii* were shown to be members of Pyrenomycetes, while *B. brongniartii* and *M. anisopliae* were members of mitosporic Ascomycota. These analyses, therefore, may reflect a close evolutionary relationship between entomopathogenic *B. brongniartii* and *M. anisopliae* and the Pyrenomycetes of Ascomycota. The clustering of *PcCHS3* from *P. chrysogenum* which belongs to the Eurotiales of Plectomycetes in the same group with *BbCHS1*, however, is contradictory to the above-mentioned prediction. Noticing that each of the two genes in the same class (class II), *PcCHS2* and *PcCHS3*, from *P. chrysogenum* is clustered into two different groups, a definitive answer on this issue will need further experiments. The bootstrap analysis and

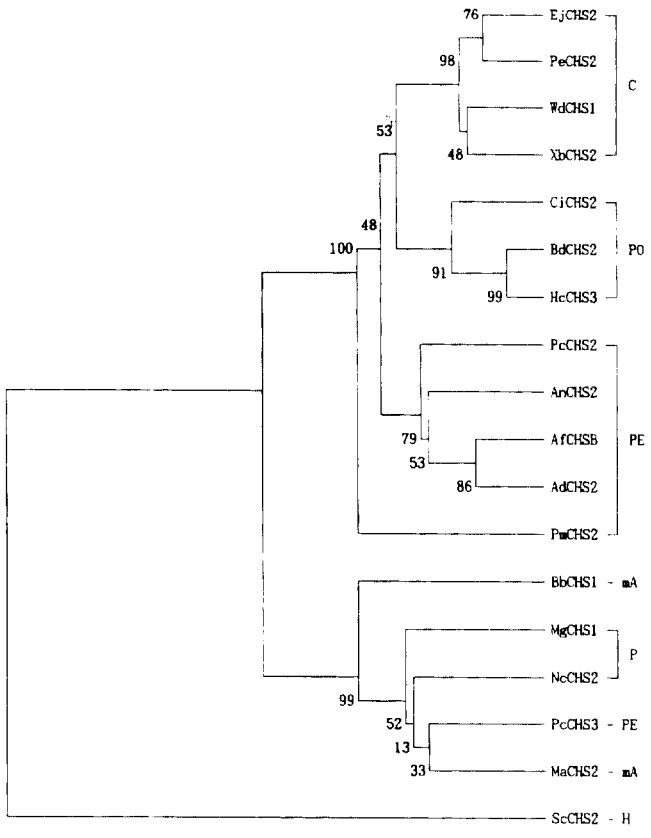

Fig. 3. Phylogenetic analysis of the deduced amino acid sequences of the class II chitin synthase. The amino acid sequences were compared by UPGMA method using MEGA ver. 1.0. Bootstrap values for 1000 replicates are reported above internal branches. C: Chaetothyriales; H: Hemiascomycetes; mA: mitosporic Ascomycota; P: Pyrenomycetes; PE: Plectomycetes Eurotiales; PO: Plectomycetes Onygenales; AdCHS2: *Emericella nidulans*; AfCHSB: *Aspergillus fumigata*; AnCHS2: *Aspergillus niger*; BbCHS1: *Beauveria brongniartii*; BdCHS2: *Blastomyces dermatitidis*; CiCHS2: *Coccidioides immitis*; EjCHS2: *Exophiala jeanselmei*; HcCHS3: *Ajellomyces capsulata*; MaCHS2: *Metarhizium anisopliae*; MgCHS1: *Magnaporthe grisea*; NcCHS2: *Neurospora crassa*; PcCHS2, PcCHS3: *Penicillium chrysogenum*; PeCHS2: *Phaeococcus exophialaes*; PmCHS2: *Penicillium marneffei*; ScCHS2: *Saccharomyces cerevisiae*; WdCHS1: *Wangiella dermatitidis*; XbCHS2: *Xylohypha bantiana*.

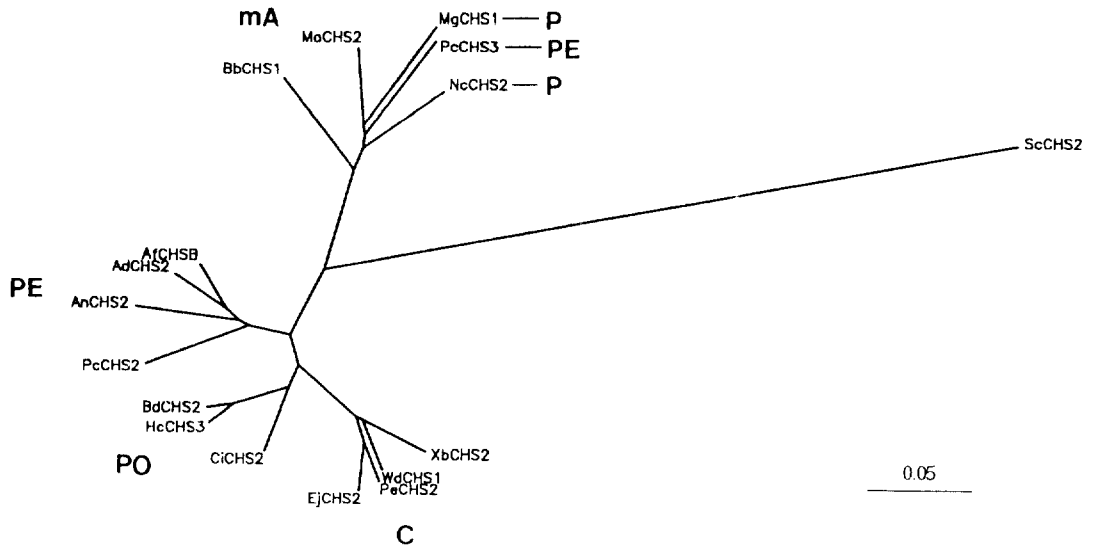

Fig. 4. Tree showing phylogenetic relationships of the class II chitin synthase fragments. The phylogenetic tree was constructed by FITCH program in Joseph Felsenstein's PHYLIP 3.5c. *S. cerevisiae CHS2* was included in the analysis as an outgroup to root the tree.

phylogenetic tree also revealed that the Onygenales of Plectomycetes are more closely related to the Chaetothyriales than to the Eurotiales of Plectomycetes. The present results clearly have shown that the PCR amplification of *CHS* fragment and subsequent analysis of its amino acid sequences could be used as a valuable key for the molecular taxonomic and phylogenetic studies of fungi. In addition, further studies on the cloning and analysis of cDNA and genomic equvalent of *BbCHS1* and its homologues will provide basic informations which will promote the practicality of *B. brongniartii* as a mycoinsecticide.

Acknowledgments

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