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Cloning and Expression Analysis of a Chitinase Gene *Crchi1* from the Mycoparasitic Fungus *Clonostachys rosea* (syn. *Gliocladium roseum*)

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***Clonostachys rosea* (syn. *Gliocladium roseum*) is a well-known biocontrol agent and widely distributed around the world. In this study, an endochitinase gene *Crchi1* was isolated from the mycoparasitic fungus *C. rosea* using the DNA walking strategy. The *Crchi1* ORF is 1,746 bp long and interrupted by three introns. The cloned gene *Crchi1* encodes 426 amino acid residues and shares a high degree of similarity with other chitinases from entomopathogenic and mycoparasitic fungi. Several putative binding sites for transcriptional regulation of *Crchi1* in response to carbon (5'-SYGGRG-3') and nitrogen (5'-GATA-3') were identified in the upstream of *Crchi1*. Expression of *Crchi1* gene in different carbon sources was analyzed using real-time PCR (RT-PCR). We found that the *Crchi1* expression was suppressed by glucose but strongly stimulated by chitin or solubilized components of the cell wall from *Rhizoctonia solani*. Phylogenetic analysis of chitinases from entomopathogenic and mycoparasitic fungi suggests that these chitinases have probably evolved from a common ancestor.**

***Keywords*:** carbon starvation, chitinase, *Clonostachys rosea*, expression, phylogenetic analysis, real-time PCR

The filamentous fungus *Clonostachys rosea* (syn. *Gliocladium roseum*: teleomorph *Bionectria ochroleuca*) is a common saprotroph with a worldwide distribution. It is associated with organisms such as fungi, nematodes and plants and is versatile in terms of its ecological niches, frequently found in cultivated soils, in wastes containing phytotoxic phenolic acids, and in aerial plant organs (Sutton *et al.,* 1997; Krauss *et al.,* 2001). Many isolates of *C. rosea* are highly efficient antagonists against several plant pathogenic fungi, as shown in the controls of *Botrytis cinerea* in strawberry, raspberry, and tomato (Sutton *et al.,* 1997), *Rhizoctonia solani* on tobacco (Lahoz *et al.,* 2002), and as an entomopathogenic fungus of insects *Oncometopia tucumana* and *Sonesimia grossa* (Toledo *et al.,* 2006). The extraordinary ecological versatility renders *C. rosea* a good candidate for use in agriculture.

As a mycoparasite, *C. rosea* has been tested successfully as a biological control agent against divergent fungal plant pathogens (Sutton *et al.,* 1997; Xue, 2003). The mycoparasitic activity has been attributed to the secretion of its cell-wall degrading enzymes, including chitinases. Chitinase (EC3.2.1.14) is a type of enzyme capable of degrading chitin. A wide variety of organisms can produce and secrete chitinase. Recent genetic evidence has suggested that a 42 kDa extracellular chitinase present in several *Trichoderma* species plays a critical role in their antagonistic activities against *B. cinerea* and *R. solani* (Lorito *et al.,* 1998; Baek *et al.,* 1999; Kim *et al.,* 2002). In addition, there is evidence that in *Trichoderma* spp. chitinase *ech42* was inducible by fungal cell walls and by colloidal chitin (Carsolio *et al.,* 1994; Garcia *et al.,* 1994) as well as by carbon starvation (Margolles-Clark *et al*., 1996). In addition, *ech42* expression appeared to be governed by carbon catabolite repression (Carsolio *et al*., 1994; Lorito *et al*., 1996).

In our previous reports, two serine proteases were identified from *C. rosea*, and both were found to play a role in infection against nematodes (Zhao *et al*., 2005; Li *et al*., 2006). However, little is known about the encoding genes of chitinase from *C. rosea* and the regulation of its expression. In this study, we described the cloning of an endochitinase gene from *C. rosea* (*Crchi1*) and the regulation of its expression using real-time PCR (RT-PCR) analysis. The phylogenetic analysis of chitinases from different organisms was also discussed.

Materials and Methods

Strains, plasmids, and growth conditions

The isolate of mycoparasitic fungus *C. rosea* used in this study was originally isolated from a field soil sample in Yunnan Province and had been deposited in China General Microbiological Culture Collection Center (CGMCC 0806). The fungal strain was grown on Potato Dextrose Agar (PDA) medium at 28°C. *R. solani* was incubated in Potato Dextrose Broth and the cell walls were prepared as described previously (Schirmböck *et al*., 1994). *Escherichia coli* DH5α, used as the recipient strain for recombinant plasmids, and was grown in Luria-Bertani medium. The vector pMD18-T (Takara, Japan) was used for PCR cloning.

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Primer design

A pair of degenerate primers (Table 1, primer set 1) was synthesized according to a previous report (Bogo *et al*., 1998) and used to amplify the conserved sequence of the chitinase gene from *C. rosea*. Two additional pairs of nested PCR primers (Table 1, primer set 2 and 3) were designed according to the amplified sequence to amplify the whole gene. A pair of specific primers containing restriction sites (*Eco*RI and *Bam*HI, respectively) (Table 1, primer set 4) were designed to amplify the cDNA sequence and for cloning. For *Crchi1* expression studies, the *Crchi1* primer (Table 1, primer set 5) was synthesized according to the *Crchi1* gene, and the beta-tubulin gene primers (*Tubb*) (Table 1, primer set 6) were synthesized based on the *C. rosea* beta-tubulin gene (AF358169) for endogenous control.

Primer dimer formation and the integrity of amplification reactions were confirmed by agarose gel electrophoresis (1.5% agarose) and melting curve analysis. In order to calculate the PCR efficiencies from *Crchi1* and *tubb* genes, a standard dilution curve was performed in duplicates using serial dilutions of standard cDNA. The efficiencies were calculated using a previously established method (Kenneth and Thomas, 2001).

Amplification of a chitinase gene from strain C. rosea CGMCC 0806

C. rosea was cultured on PDA medium for 7 days at 28°C. Mycelium for DNA extraction was collected from the plates and ground to a fine powder in liquid nitrogen. DNA was extracted using the CTAB method described previously (Zhang *et al*., 1996).

A pair of degenerate primers (Table 1, primer set 1) was used to amplify the conserved sequence of chitinase genes from *C. rosea*. The genomic DNA was used as templates, and PCR conditions followed those described in a previous report (Yang *et al*., 2005). To obtain the complete chitinase gene from *C. rosea*, two pairs of nested PCR primers were designed targeting the conserved sequence. The 5' and 3' terminal fragments of the chitinase gene were amplified using the DNA Walking Speedup™ Premix Kit (Seegene, Korea), separately. PCR amplification was performed according to previous report (Gan *et al*., 2007). All PCR products were purified and sequenced using an ABI PRISM 3730 automated sequencer (Perkin-Elmer, USA) with four fluorescent dyes.

Sequence analysis

Analysis of the DNA sequence was done using the DNAman software package (Version 5.2.2, Lynnon BioSoft, Canada). Homology search was performed using the BLAST algorithms against various databases in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Potential N-glycosylation sites were analyzed by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Molecular weight, theoretical pI and amino acid composition were analyzed by ProtParam tool (http://us.expasy.org/tools/protparam.html).

Gene expression analysis

For *Crchi1* expression studies, *C. rosea* was first precultured for 3 days on a rich medium (Potato Dextrose Broth). The mycelium was collected by filtration, washed three times with sterilized water and transferred to liquid synthetic medium (SM) as described previously (Robert *et al*., 1999). The carbon source in the synthetic medium was glucose, colloidal chitin, cell wall components of *R. solani* or periostracum cicadae. Mycelium was harvested after 48 h of incubation for RNA extraction.

Total RNA was isolated using the RNAprep Plant Kit (DP402 TianGen, China) according to the manufacturer's protocol. Integrity of RNA was confirmed by running samples in a 1% agarose gel. RT-PCR amplification was performed using the RNA PCR Kit (AMV) Ver. 2.1 (TaKaRa, Japan) according to the manufacturer's protocol.

Expression levels of *Crchi1* were quantified with a real-time RT-PCR assay performed in an ABI Prism 7000 sequence detection system (Applied Biosystems, USA), using the SYBR Green Jump Start Taq Ready Mix (Sigma, USA). Each 20 μl reaction mixture consisted of 10 μl SYBR Green Supermix, 1 μl of each primer at 10 μmol, 6 μl nuclease-free water and 2 μl diluted cDNA. Synthesis of cDNA and

Table 1. List of oligonucleotide primers used in this study

	Primers	Sequence (5'-3')
1	Forward	GC(TC) GT(TC) TA(TC) TT(TC) AC(TC) AA(TC) TGG
	Reverse	GG(AG) TA(TC) TCC CA(AG) AT(AG) TC(CGAT) AT
2	Forward TSP1	TGC CCC TGA AAA ATG GAT GA
	Forward TSP3	CAT GAA TGG TCA CGA TGG ACT AAC
3	Reverse TSP1	CAT TGG TGG CTG GAC TTG GT
	Reverse TSP3	GCT GTT GAG TTC ATG AAG GAT TGG
4	Former	CCGGAATTCATG AAG TCG TTT TTC ACC CG
	Reserve	CGCGGATCCTCA GGA GAG GCT GTT CTT G
5	Crchi1F	CTC AAC TCC ATT CAG CAC CAA G
	Crchi1R	TGT AGT CCC AGA TTC CGC TCT C
6	TubbF	CAT CTT CAG ACC GGT CAG TG
	TubbR	AAG TAG ACG TTC ATG CGC TC

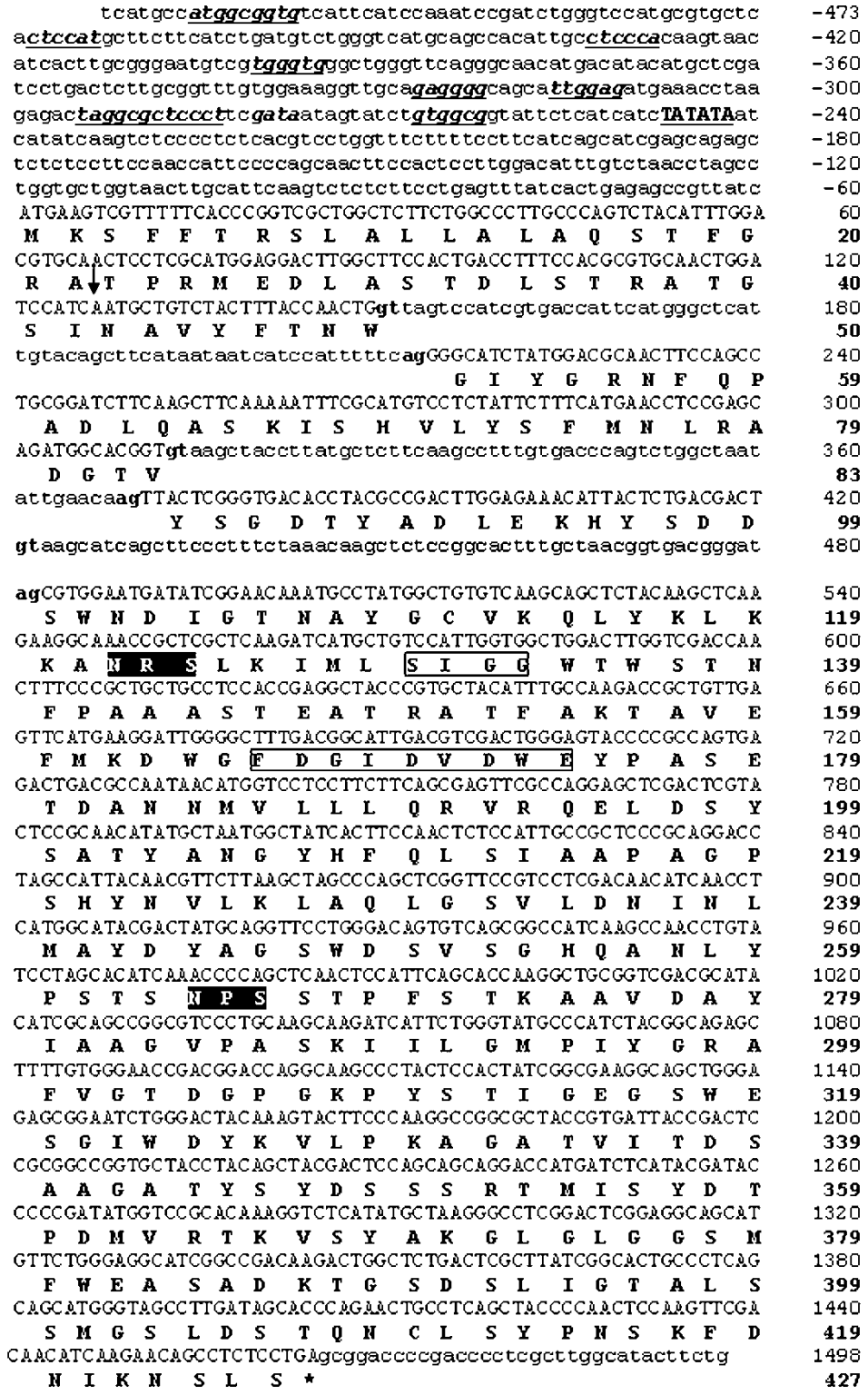

Fig. 1. Nucleotide sequence of the *Crchi1* from *C. rosea* (GenBank accession no. EU000575). The deduced amino acid sequence is shown in one-letter code under the DNA sequence. Nucleotide coordinates are shown to the right of the sequence. Introns are shown in lower-case letters, and their conserved 5'GT, 3'AG are in bold. General transcription control signals including TATAA regions upstream of the gene are in bold and underlined. Putative transcription control sites within the promoter region for carbon regulation (5'-SYGGRG-3') are in bold italics and underlined, sites for nitrogen regulation (5'-GATA-3') are in bold italics. The possible signal sequence cleavage point is represented by vertical arrows between the A_{22} and T_{23} residues. Potential N-glycosylation sites are by NRS and NPS residues and shaded in black. The white boxes (amino acids 130-133 and 166-174) corresponds to the conserved motif from family 18 of the glycosyl hydrolases.

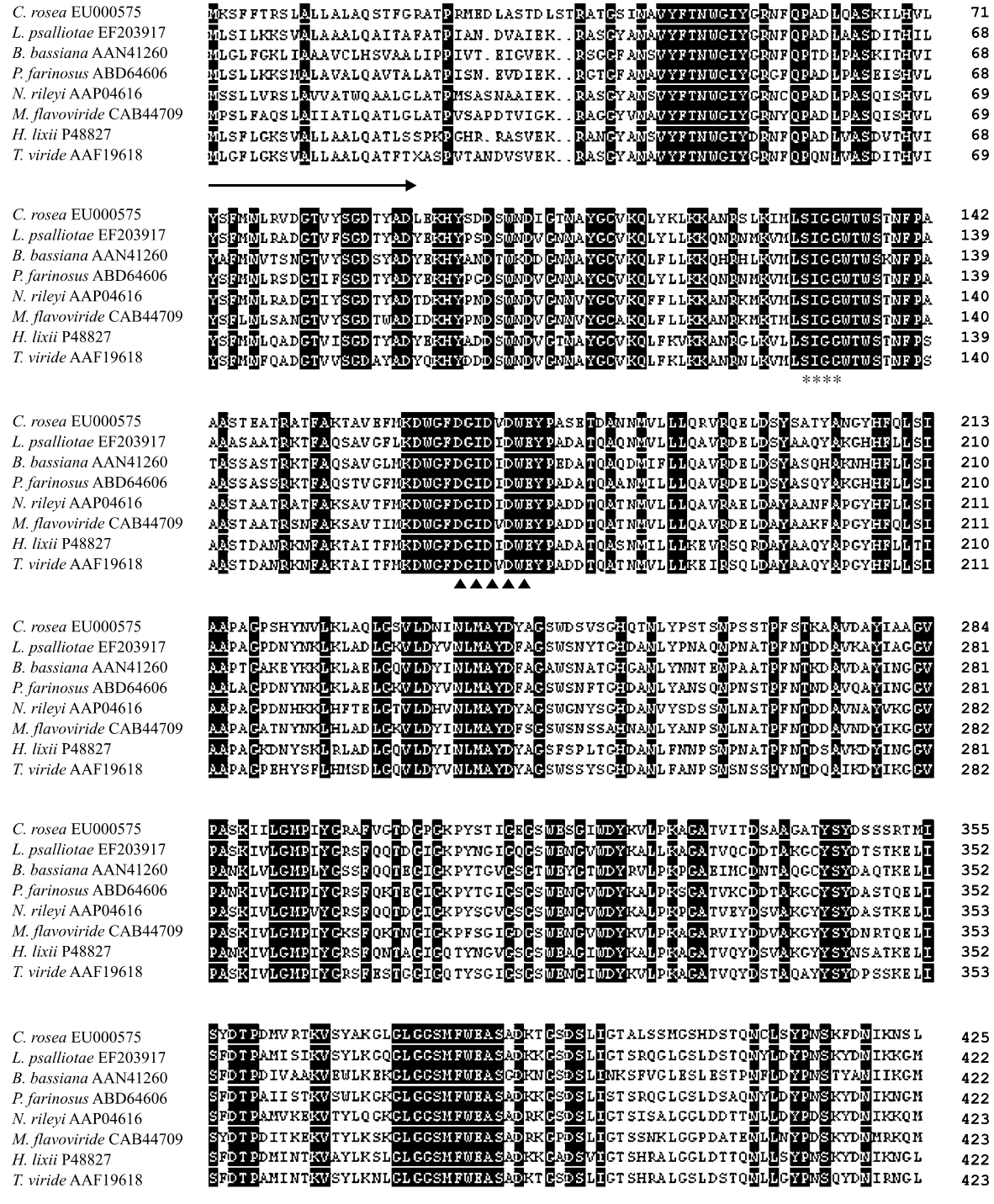

Fig. 2. Alignment of the amino acid sequences of chitinases from different fungi. Areas shaded in black are conserved regions (100% similarity), and unshaded areas are regions of variability between the chitinases. Signal peptide sequences are marked on arrow, *** indicated the conserved substrate binding sites (SXGG), and ▲ indicated the catalytic domains (DXXDXDXE) of glycosyl hydrolase family 18.

RT-PCR was performed in triplicates for each gene. The PCR conditions followed the manufacturer's instructions. RT-PCR reactions for the endogenous control beta-tubulin gene (*Tubb*) and the target gene *Crchi1* were conducted in separate tubes. The expression level of *Crchi1* grown for 48 h on 1% glucose was used as the calibrator and defined as one. The expression level calculated by the formula $2^{-\triangle\triangle CT}$ represents the x-fold difference from the calibrator.

Determination of fungal biomass

Fungal biomass was determined using a method described previously (Robert *et al*., 1999). Values were in milligrams

of extractable protein per milliter of culture.

Phylogenetic analysis

Amino acid sequences of chitinases from different organisms were aligned with CLUSTAL W (Thompson *et al*., 1994) and a phylogenetic tree was constructed using PAUP version 4.0b10 (Swofford, 2002). Confidence values for individual branches were assessed from 1000 bootstrap replicates of the original sequence data.

Results

Sequence analysis of the chitinase gene Crchi1 from C. rosea CGMCC 0806

A 579 bp PCR product was amplified by using degenerate primers set 1 (Table 1). The 5' and 3' flanking sequences were amplified using the DNA Walking Speedup™ Premix Kit, separately. The complete chitinase gene was obtained from the assembled DNA sequence with the DNAman software package. The sequence was verified as a chitinase gene by homologous analysis with other fungal chitinase genes. The complete nucleotide sequence of *Crchi1* has been submitted to GenBank (accession number EU000575).

The encoding gene of CrChi1 consisted of one open reading frame (ORF) and three short introns (64, 58, and 62 bp in length, respectively). The splice junctions were determined by comparing the sequence of *Crchi1* with that of its corresponding cDNA sequence. Each intron began with GT and ended with AG, a common feature of fungal introns (Gurr *et al*., 1987). The elements commonly found within fungal promoters were identified (Fig. 1) and include a TATAA box located at 188 bp upstream of the translation start codon ATG. In addition, putative binding sites for transcriptional regulation of *Crchi1* in response to carbon (binding site 5'-SYGGRG-3') (Sophianopoulou *et al*., 1993) and nitrogen (binding site 5'-GATA-3') (Kudla *et al*., 1990) are presented in Fig. 1.

The chitinase gene *Crchi1* encodes a polypeptide of 426 amino acid residues. Homologous analysis indicates that CrChi1 is synthesized as a preproenzyme, and the first 22 amino acids have many characteristics of a signal peptide and the signal peptidase cleavage site is between A_{22} and T_{23}. After processing of the 22 N-terminal amino acids, the calculated size of CrChi1 is 43.8 kDa. Two potential N-glycosylation sites (NRS and NPS) were found by computational prediction (Fig. 1). Comparison of the CrChi1 amino acid sequence with other chitinases in the GenBank database revealed that they shared a high degree of similarity among fungal chitinases, and the regions of SIGGW and FDGIDVDWE were highly conserved among those chitinases of the glycosyl hydrolase family 18 (Fig. 2). The active site centered on the catalytic residues Asp_{167}, Asp_{170}, Asp_{172} and Glu_{174} (in CrChi1) are conserved in all aligned sequences. Previous studies have indicated that substitution at any of these sites (D or E) could result in greatly reduced activity or a total elimination of activity (Seur *et al*., 2007).

Moreover, the chitinase gene *Crchi1* shared a high degree of similarity with another endochitinase gene *Crech42* from *C. rosea*. The percent sequence identity was 85.12% at the nucleotide sequence ORF level (GenBank accession no. DQ523687) and 81.69% at the deduced amino acid sequence level (GenBank accession no. ABG02418). *Crchi1* contained three short introns, while *Crech42* contained only two introns.

Gene expression analysis

Amplification using the *Crchi1* primer (Table 1, primer set 5) yielded a 179 bp product, and the *tubb* primers (Table 1, primer set 6) yielded a 137 bp product. When analyzing the melting curve, a single product-specific melting curve was obtained for primers of *Crchi1* and *tubb*, respectively, indicating that primers were designed with optimal efficiency and were effective at targeting and amplifying only the genes of interest (data not shown). A plot of the log cDNA dilution versus $\triangle C_T$ ($C_{T,\ Crchi1}$-$C_{T,\ Tubb}$) showed that the absolute value of the slope is close to 0.10, suggesting that the efficiencies of the target gene and the reference gene were similar. We therefore used the $\triangle\triangle C_T$ calculation for the relative quantifications of the target gene.

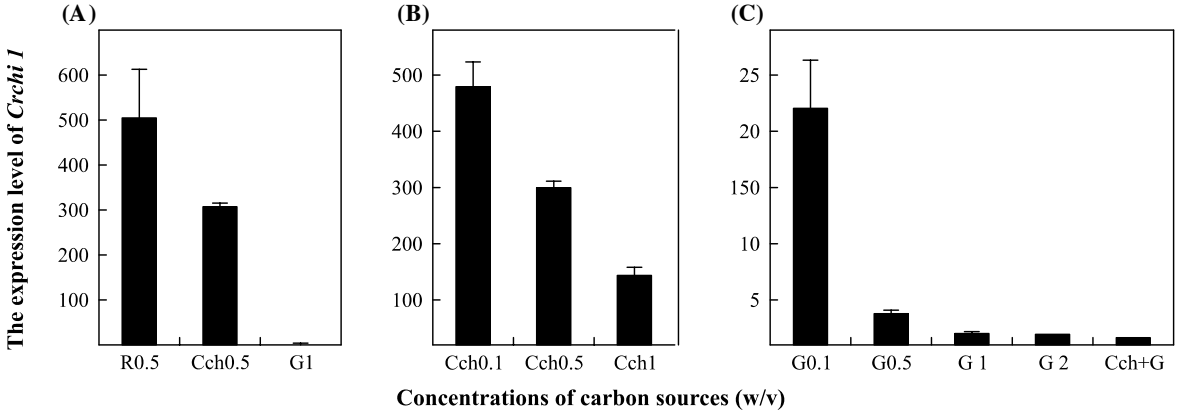

Fig. 3. RT-PCR analyses of the expression of *Crchi1* in *C. rosea*. Strains were cultivated on SM with different C sources [colloidal chitin (Cch), glucose (G), and *R. solani* cell walls (CWRs or R)] for 48 h. The numbers 0.1, 0.5, 1 and 2 in X axis represent the different concentrations [weight in volume (w/v)] of carbon sources. A housekeeping gene, beta-tubulin (*Tubb*) was used as the endogenous control to help quantify PCR products. The expression level of *Crchi1* grown for 48 h on 1% glucose was used as the calibrator and defined as one. The expression level calculated by the formula $2^{-\triangle\triangle CT}$, Y axis represents the x-fold difference from the calibrator. Error bars indicate SD.

Quantitative analysis of expression levels of *Crchi1* was achieved by a RT-PCR assay (Fig. 3). Our results showed that both genes (*Crchi1* and *tubb*) were found to be expressed constitutively. The highest level of *Crchi1* expression was observed with 0.5% CWRs (cell walls of *R. solani*) (Fig. 3A), followed by 0.5% CCh (colloidal chitin) (Fig. 2A and B), and 1% G (glucose) (Fig. 3A and C). The induction of *Crchi1* by the different concentrations of glucose and colloidal chitin were also tested (Fig. 3B and C). Glucose induced the highest level of expression at the concentration of 0.1% and the lowest level of expression at 2% (Fig. 3C). Similarly, CCh induced the highest level of expression at the concentration of 0.1% and the lowest level of expression at 1% (Fig. 3B). *Crchi1* mRNA showed a relative low level expression on 1% glucose in the presence of the 0.5% CCh (Fig. 3C).

Furthermore, with respect to *C. rosea* growth in SM with different concentrations of glucose and colloidal chitin, fungal biomass was determined (Fig. 4). The biomass results contrasted those of *Crchi1* expression. Specifically, biomass formation was lowest grown on 0.1% glucose and colloidal chitin. These results suggested that carbon starvation induced *Crchi1* expression in *C. rosea*, consistent with previous reports (Margolles-Clark *et al*., 1996; Robert *et al*., 1999).

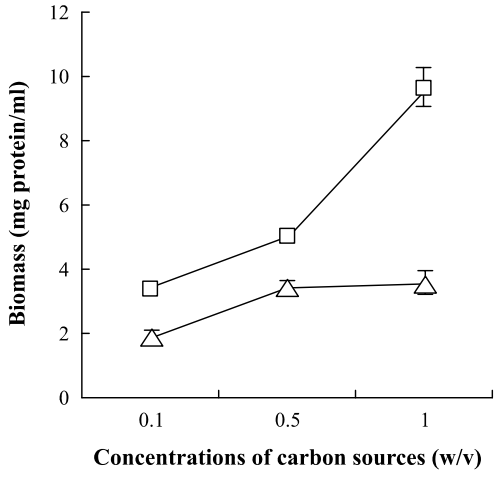

Fig. 4. Biomass formation (total extractable protein) of *C. rosea* during cultivation on different concentration of glucose (□) and colloidal chitin (△). Values are means of at least three separate experiments carried out with the spores (6×10^6/ml), error bars indicate standard deviation.

Phylogenetic analysis

The phylogenetic tree of chitinases (Fig. 5) from various organisms was constructed using PAUP version 4.0b10 (Swofford, 2002). One chitinase belonging to glycosyl hydrolase family 19, identified from *Zea diploperennis* (Tiffin, 2004) was used as the outgroup. Other chitinases all belonged to glycosyl hydrolase family 18. In family 18, chitinases from *Neurospora crassa*, *Botryotinia fuckeliana*, *Aspergillus fumigatus* and *Coniothyrium minitans* were clustered and formed a clade (clade II), while other chitinases from entomopathogenic and mycoparasitic fungi were clustered and formed a monophyletic clade (clade I). The monophyletic lineages provided a support for the hypothesis that these chitinases likely evolved from one common ancestor. Moreover, clade I consisted of three subclades: chitinases from mycoparasitic fungi (*Hypocrea* spp., *Trichoderma* spp.) were clustered and formed the subclade Ia, chitinases from different entomopathogenic fungi (e.g. *Beauveria bassiana*, *Paecilomyces fumosoroseus*, *Metarhizium anisopliae*) were clustered and formed the subclade Ib, and chitinase CrChi1 and CreCh42 from *C. rosea* formed the subclade Ic, and there is a statistical support (54%) for the significant divergence of CrChi1 from other chitinases.

Interestingly, the topology of the phylogenetic tree co-

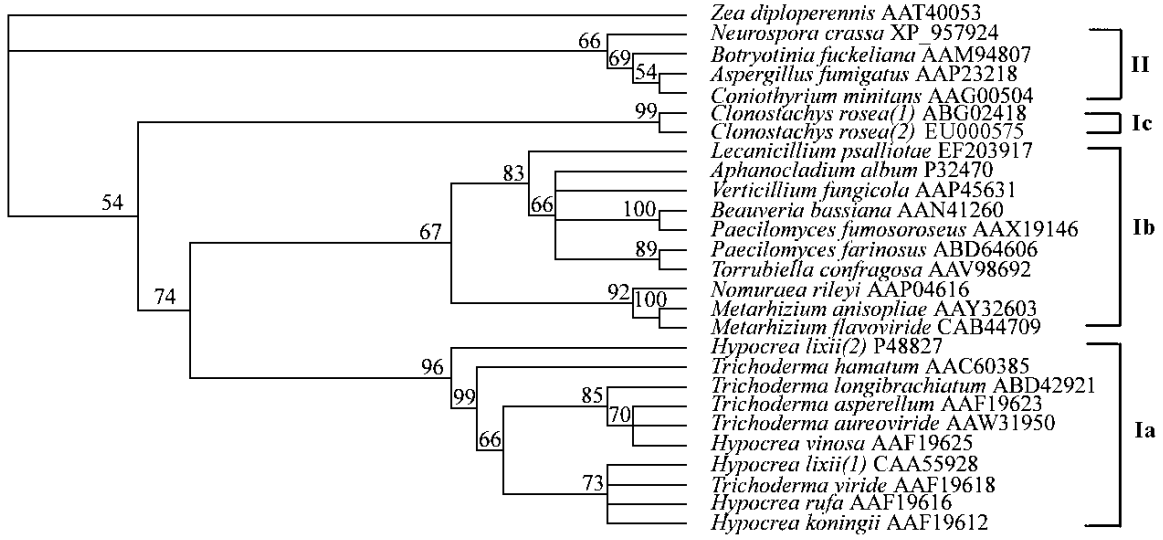

Fig. 5. Phylogenetic tree showing the relationship between CrChi1 and other chitinases. The tree was constructed using the PAUP version 4.0b10 (Swofford 2002) program packages. The chitinase gene of *Z. diploperennis* (AAT40053) was used as an outgroup to root the tree. Confidence values were assessed from 1,000 bootstrap replicates of the original sequence data.

Table 2. Chitinases from different organisms used in our phylogenetic analysis

Taxonomy	Name	Accession no.
Eukaryota		
Fungi		
Ascomycota		
Eurotiomycetes		
Eurotiales		
Trichocomaceae		
Aspergillus	*Aspergillus fumigatus*	AAP23218
Leotiomycetes		
Helotiales		
Sclerotiniaceae		
Botryotinia	*Botryotinia fuckeliana*	AAM94807
Dothideomycetes		
Pleosporales		
Leptosphaeriaceae		
Coniothyrium	*Coniothyrium minitans*	AAG00504
Sordariomycetes		
Sordariales		
Sordariaceae		
Neurospora	*Neurospora crassa*	XP_957924
mitosporic Ascomycota		
Aphanocladium	*Aphanocladium album*	P32470
Sordariomycetes		
Hypocreales		
Bionectriaceae		
Bionectria	*Clonostachys rosea* (1)	ABG02418
	Clonostachys rosea (2)	EU000575
Clavicipitaceae		
Lecanicillium	*Lecanicillium psalliotae*	EF203917
Beauveria	*Beauveria bassiana*	AAN41260
Paecilomyces	*Paecilomyces fumosoroseus*	AAX19146
	Paecilomyces farinosus	ABD64606
Torrubiella	*Torrubiella confragosa*	AAV98692
Nomuraea	*Nomuraea rileyi*	AAP04616
Metarhizium	*Metarhizium anisopliae*	AAY32603
	Metarhizium flavoviride	CAB44709
mitosporic Hypocreales		
Verticillium	*Verticillium fungicola*	AAP45631
Trichoderma	*Trichoderma hamatum*	AAC60385
	Trichoderma longibrachiatum	ABD42921
	Trichoderma asperellum	AAF19623
	Trichoderma aureoviride	AAW31950
	Trichoderma viride	AAF19618
Hypocreaceae		
Hypocrea	*Hypocrea lixii* (1)	CAA55928
	Hypocrea lixii (2)	P48827
	Hypocrea koningii	AAF19612
	Hypocrea rufa	AAF19616
	Hypocrea vinosa	AAF19625

incided with the taxonomic status of these fungi, except two species *Aphanocladium album* (P32470) and *Verticillium fungicola* (AAP45631) (Table 2). All fungi analyzed here were from the same class Ascomycetes. Fungi in the first subgroup (clade I) belong to the order Hypocreales and comprises several families (Bionectriaceae, Clavicipitaceae, Hypocreaceae) that correspond to their additional groupings based on the chitinase sequences. The second subgroup (clade II) contained fungi from families Sordariales of the order Sordariomycetes (*N. crassa*), and from Orders

Leotiomycetes (*B. fuckeliana*), Eurotiomycetes (*A. fumigatus*) and Dothideomycetes (*C. minitans*).

Discussion

Clonostachys rosea displayed strong antagonistic activities against many soil and root-dwelling fungi. The success of *C. rosea* as a biocontrol agent is believed to involve many factors and diverse modes of action. The hydrolytic enzymes (e.g. protease and chitinase) produced by *C. rosea* likely play a key role in its ability to penetrate and kill a host. In this study, we cloned a chitinase gene (*Crchi1*) from *C. rosea* using the DNA walking technology. Comparison of the 5'-flanking DNA sequences of co-regulated genes may help identify conserved regulatory sequences (Gwynne *et al.*, 1987). Such regions may include binding sites for either general or special transcription regulatory proteins. Thus, this strategy was used to identify promoter elements that might be involved in the coordinated expression of *Crchi*. The *Crchi1* promoter displayed eight consensus sites (5'-SY GGRG-3') for binding of the carbon catabolite repressor CreA (Sophianopoulou *et al*., 1993). These sites are located at the upstream of the TATA box. One GATA site is present in the *Crchi1* promoter. The GATA sequences are recognized by the major positive regulatory elements AreA (in *Aspergillus nidulans*) and Gln3 (in *Saccharomyces cerevisiae*) (Kudla *et al*., 1990; Minehart and Magasanik, 1991). Both AreA and Gln3 mediate global nitrogen repression and derepression. The role of these sequences in the regulation of *Crchi1* is unknown and requires further investigation.

A high level *Crchi1* expression was observed in medium with 0.5% CWRs (cell walls of *R. solani*) (Fig. 3A). This result suggests that that a soluble component from the cell walls may act as an inducer for *Crchi1* expression. *C. rosea* has been reported as an efficient antagonist against *R. solani* on tobacco (Lahoz *et al*., 2002). Therefore, chitinase CrChi1 may play a role during the interaction between *C. rosea* and the phytopathogen *R. solani.* In addition, 0.5% CCh (colloidal chitin) also induced a high level expression of *Crchi1*. Because purified colloidal chitin used in our assays is significantly different from the naturally occurring polymer in fungal cell walls, therefore, their induction potentials could also be very different. However, the expression of *Crchi1* could not be detected when the strain was grown in 0.5% periostracum cicadae, even though *C. rosea* was reported as an entomopathogenic fungus of *Oncometopia tucumana* and *Sonesimia grossa* (Toledo *et al*., 2006). These results suggest other hydrolytic enzymes (e.g. protease) must be playing a significant role during infections against these hosts. In contrast, cultures grown in 1% glucose had a low level *Crchi1* mRNA. This was likely due to the lack of an inducer or the presence of a suppressor, since the promoter region of *Crchi1* contains putative binding sites for the regulatory protein CreA. The expression of *Crchi1* decreased with increasing glucose concentrations (Fig. 3B). In addition, our results showed that *Crchi1* mRNAs accumulated in fungi grown on 1% glucose in the presence of the 0.5% CCh (colloidal chitin), but reaching a low expression level. The low level expression suggests that glucose have a suppressive effect on the expression of *Crchi1*. Similarly, the expression of *Crchi1* also decreased following the increase of colloidal chitin (Fig. 3C).

Several chitinases have been identified from the fungal species *Trichoderma* sp. (Seidl *et al*., 2005). However, only the 42 kDa (Lorito *et al*., 1998) and the 33 kDa proteins (Limon *et al*., 1999) were found inducible to higher biological activity levels. In this study, the 42 kDa chitinases derived from various organisms were selected to investigate the evolutionary relationships between CrChi1 and other chitinases. Interestingly, except two species *A. album* and *V. fungicola*, the topology of the chitinase phylogenetic tree was very similar to the taxonomic relationships among these fungi inferred based on their structural and reproductive features. The incongruence for two of the species is understandable because some fungal species are highly pleomorphic and have anamorpich life cycles with widely divergent types of propagations (De Hoog *et al*., 1994). Species in some of the genera cannot be identified using morphological characters alone. Additional physiological tests and sequence analyses may be needed. Recently, Schickler *et al*. (1998) developed an electrophoretic method using chitinases (Ech42) as a marker for identifying species in the genus *Trichoderma*. Therefore, the 42 kDa chitinases derived from ascomycetes may serve as a reference marker in future phylogenetic analyses. Moreover, the redundancy of the chitinase genes within the same species may reflect their functional differences between related proteins (Orikoshi *et al*., 2003) and the adaptive evolution of the chitinases.

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