Identification of mycoparasitism-related genes in *Clonostachys rosea* 67-1 active against *Sclerotinia sclerotiorum*

Zhan-Bin Sun, Man-Hong Sun & Shi-Dong Li

*Clonostachys rosea* is a mycoparasite that has shown great potential in controlling various plant fungal pathogens. In order to find mycoparasitism-related genes in *C. rosea*, the transcriptome of the efficient isolate 67-1 in association with sclerotia of *Sclerotinia sclerotiorum* was sequenced and analysed. The results identified 26,351 unigenes with a mean length of 1,102 nucleotides, among which 18,525 were annotated in one or more databases of NR, KEGG, Swiss-Prot, GO and COG. Differentially expressed genes at 8 h, 24 h and 48 h after sclerotial induction were analysed, and 6,890 unigenes were upregulated compared with the control without sclerotia. 713, 1,008 and 1,929 genes were specifically upregulated expressed, while 1,646, 283 and 529 genes were specifically downregulated, respectively. Gene ontology terms analysis indicated that these genes were mainly involved in metabolism of biological process, catalysis of molecular function and cellular component. The expression levels of 12 genes that were upregulated after encountering with *S. sclerotiorum* were monitored using real-time PCR. The results indicated that the quantitative detection was consistent with the transcriptome analysis. The study provides transcriptional gene expression information on *C. rosea* parasitizing *S. sclerotiorum* and forms the basis for further investigation of mycoparasitism-related genes of *C. rosea*.

*Sclerotinia sclerotiorum* (Lib.) de Bary is a destructive fungal pathogen that infects more than 450 species of plants worldwide and causes huge economic losses in some important agricultural crops and vegetables, such as soybean, rape and carrot. In most cases, *S. sclerotiorum* exists in infected plants, seeds and soil in the form of sclerotia which can survive for a long time. Under suitable temperature and humidity conditions, the sclerotia germinate and infect the leaves, stems and fruit, or infect plants directly through mycelium. Therefore, the pathogen *S. sclerotiorum* is hard to eliminate.

The control of Sclerotinia diseases includes pesticides, agricultural practices and biopesticides, among which biocontrol has received more attention because of its environmental and food security advantages. *Clonostachys rosea* is a mycoparasite of various plant pathogenic fungi and has shown great potential to control numerous plant diseases. However, the molecular mechanism of mycoparasitism of the fungus has not yet been clarified.

Mycoparasitism is an important method for biocontrol fungi against fungal pathogens. The mycoparasites attach to hyphae of the pathogens, suppress extension of germ tubes, and cause mycelial deformity. Tu observed the interaction of *Gliocladium virens* and *S. sclerotiorum* by using electron microscopy and revealed that the mycoparasite formed appressorium-like structures on the host fungus, penetrated and parasitized internal cells of sclerotia. Similar structures were also detected in *Trichoderma* spp. and *C. rosea* during their parasitic processes. Up to now, several mycoparasitism-related genes have been identified and verified, many of which are cell wall degrading enzymes and were isolated from *Trichoderma* spp. The disruption of endochitinase and β-1,6-glucanase encoding genes in *T. virens* remarkably reduced the control efficiencies against pineapple and cotton diseases, while overexpression of the extracellular serine protease gene *tvsp1* increased the ability to protect cotton seedlings against *Rhizoctonia solani*. Malmierca et al. isolated a cytochrome P450 monooxygenase encoding gene *tri4* and a terpene synthase gene *tri5* which were critical in producing trichothecene harzianum A from *T. arundinaceum*, and found that the mutants deleted for *tri4* and *tri5* led to a drastic reduction of antifungal activity.
activity against R. solani and Botrytis cinerea. Cloning and analysis of differently expressed genes in Coniothyrium minitans during the mycoparasitic process against S. sclerotiorum were conducted, which identified genes encoding β-1,3-glucanase\textsuperscript{15,16}, and those associated with MAP kinase cascade\textsuperscript{17} and heat shock factors\textsuperscript{18}.

Several mycoparasitism-related and biocontrol-related genes of C. rosea have also been reported. Kosawang et al.\textsuperscript{19} disrupted a zearalenone encoding gene, \textit{zhd101}, from C. rosea, which led to the loss of zearalenone detoxification ability and a weak pathogenicity to the zearalenone-producing fungus Fusarium graminearum. Mouekouba et al.\textsuperscript{20} demonstrated that two plant resistance related factors, WKRY and MAPK, were upregulated in \textit{C. rosea} during its interaction with \textit{B. cinerea}, and were involved in the suppression of tomato grey mould by inducing plant resistance. Zou et al.\textsuperscript{21} indicated that mutants deficient for the subtilisin-like extracellular serine proteases gene \textit{prC} attenuated virulence to nematodes. Some proteins associated with physiology and metabolism of the microorganism, e.g. zinc finger transcription factor and ATP-binding cassette transporter, were also confirmed to be involved in the parasitic process of the fungus against nematodes and pathogenic fungi\textsuperscript{22,23}. However, the molecular mechanisms by which \textit{C. rosea} parasitizes \textit{S. sclerotiorum} are unclear. In previous study, a microscopic observation of sclerotia section of \textit{S. sclerotiorum} treated by \textit{C. rosea} 67-1 was performed, revealing that the hypha of the mycoparasite penetrated into the sclerotia and the tissue of the fungal host collapsed\textsuperscript{24}. In this paper, the transcriptome of the isolate 67-1 parasitizing sclerotia was sequenced and analysed to find mycoparasitism-related genes. The results will provide a valuable foundation for further exploration of functional genes and molecular mechanism of \textit{C. rosea} against \textit{S. sclerotiorum}.

### Results

**De novo transcriptome assembly.** The transcriptome of \textit{C. rosea} 67-1 under sclerotial induction and on PDA medium was sequenced using the Illumina HiSeq 2000 platform. 181,076,576 raw reads were obtained from the six mycelia samples collected in different alimentation modes and stages. After filtering low quality reads and adaptor sequences, 14,468,003,640 bp of high quality clean reads remained and were further assembled. The Q\textsubscript{50} values of the six samples were higher than 95.5%, and the GC contents were 53.7–55.6%. The clean data were assembled de novo using the Trinity software and 26,351 unigenes, comprising 29,036,163 bp, were obtained, including 6,526 distinct clusters and 19,825 distinct singletons, with a mean length of 1,102 bp and an N\textsubscript{50} of 1,861 bp. The contigs and unigenes of different samples were analysed statistically, and only 1,779 (6.75%) unigenes were longer than 3,000 bp (Table 1).

### Functional annotation and classification of the 67-1 transcriptome.

Functional annotation of all the unigenes was conducted, and a total of 18,525 unigenes (70.2%) could be annotated in one or more databases, among which 18,324 (98.9%), 11,371 (61.4%), 11,595 (62.6%), 8,615 (46.5%) and 8,808 (47.5%) were annotated to the NR, Swiss-Prot, KEGG, COG and GO databases, respectively, with an E-value < 10\textsuperscript{−5}. The remaining 7,862 unigenes (29.8%) that lacked annotation information might represent new genes. Using Blast and ESTscan software, coding the sequences (CDGs) of 19,380 unigenes were successfully predicted.

The distribution of E-values in the NR annotation indicated that 50.2% of the unigenes had a high homology, with E-value less than 10\textsuperscript{−60}, and the similarity distribution indicated that 54.7% of the unigenes had a similarity higher than 60%. More than half of the annotated genes were related to parasitic fungi, plant pathogens or biocontrol fungi. 20.8% were associated with \textit{Nectria haematococca} and 11.3% with \textit{F. oxysporum} Fo5176; and 5.1% with \textit{F. pseudograminearum} CS3096. A number of unigenes matched with genes from biocontrol fungi such as \textit{M. anisopliae} ARSEF 23 (5.9%), \textit{M. acridum} CQMa 102 (5.2%), \textit{T. virens} Gv29-8 (5.6%) and \textit{T. atroviride} IMI 206040 (4.5%) (Fig. 1).

### Table 1. Statistics of the assembly quality of the transcriptome of \textit{C. rosea} 67-1.

| Sample/Contig | Total number | Total length (bp) | N\textsubscript{50} | E-value | DC\textsuperscript{2} | DS\textsuperscript{4} |
|---------------|--------------|--------------------|----------------|---------|----------------|----------------|
| 8a            | 48,302       | 20,160,931         | 974            | —       | —              | —              |
| 8b            | 46,601       | 20,726,522         | 1079           | —       | —              | —              |
| 24a           | 46,550       | 19,010,037         | 932            | —       | —              | —              |
| 24b           | 47,530       | 21,894,686         | 1191           | —       | —              | —              |
| 48a           | 39,178       | 16,046,611         | 958            | —       | —              | —              |
| 48b           | 39,245       | 19,122,207         | 1283           | —       | —              | —              |
| Total Contig  | 26,351       | 29,036,163         | 1861           | —       | —              | —              |

| Sample/Unigene | Total number | Total length (bp) | N\textsubscript{50} | E-value | DC\textsuperscript{2} | DS\textsuperscript{4} |
|----------------|--------------|--------------------|----------------|---------|----------------|----------------|
| 8a             | 30,247       | 20,470,590         | 1105           | —       | 3,980          | 26,267         |
| 8b             | 30,165       | 20,243,658         | 1084           | —       | 3,025          | 27,140         |
| 24a            | 29,111       | 19,261,908         | 1063           | —       | 3,730          | 25,381         |
| 24b            | 29,521       | 21,923,337         | 1248           | —       | 3,555          | 25,966         |
| 48a            | 25,810       | 16,243,591         | 1069           | —       | 2,790          | 23,020         |
| 48b            | 26,270       | 19,603,112         | 1323           | —       | 2,974          | 23,296         |
| Total Unigene  | 26,351       | 29,036,163         | 1861           | —       | 6,526          | 19,825         |
The functions of 8,615 unigenes annotated in the COG database were predicted and classified into 25 categories. The largest group was 'general function prediction only', representing 40.5% of the COG-annotated unigenes. 'Transcription' was second, representing 24.2% of total unigenes, followed by the groups 'carbohydrate transport and metabolism' (22.1%), 'function unknown' (18.6%), and 'translation, ribosomal structure and biogenesis' (18.2%) (Fig. 2).

The functions of the unigenes were further predicted using GO assignments, a standard system for gene function classification. Among its three ontologies, 18,895 unigenes matched 20 terms in the biological process group, 11,166 unigenes matched 14 terms in the cellular component group, and 10,455 unigenes matched 14 terms in the molecular function group. Most unigenes were associated with physiological and metabolic processes in the living organism; however, several genes might be related to its interaction with other fungi, though the number of these categories was much low. For example, seven unigenes were involved in 'locomotion' and 'biological adhesion'. A number of unigenes with the features of 'catalytic activity' and 'binding function' might also play important roles in different nutrition modes of the biocontrol fungus (Fig. 3).

To analyse gene products associated with intracellular metabolic processes, the KEGG pathway database was used and 11,595 unigenes involved in 108 KEGG pathways were identified. The analysis indicated that 'metabolic pathways', 'biosynthesis of secondary metabolites' and 'starch and sucrose metabolism' were the dominant pathways active during C. rosea 67-1's recognition and parasitization of S. sclerotiorum: the proportions reached 31.6%, 13.4% and 8.9% respectively. However, some pathways were associated with very few unigenes: 'C5-branched dibasic acid metabolism' (six), and 'lipoic acid metabolism' (three) and 'caffeine metabolism' (three) (Table 2).

Identification of differentially expressed genes (DEGs) of C. rosea 67-1. The gene expressions of 67-1 parasitizing on sclerotia and vegetative growth on PDA medium were compared at each time point and 10,504 DEGs were detected in the two growth conditions (Fig. 4). During the parasitic process, a total of 6,890 unigenes were upregulated, among which 604 rose continuously over the time course, and 713 DEGs were early stage-specific (0–8 h), 1,008 were middle stage-specific (8–24 h), and 1,929 were late stage-specific (24–48 h). Meanwhile 3,614 genes were found downregulated, including 282 continued falling genes and 1,646, 283 and 529 specific DEGs at 8 h, 24 h and 48 h, respectively. At the beginning of parasitism, numerous of unigenes changed their expression dynamically under sclerotial induction; 3,217 genes were upregulated and 2,739 were downregulated compared
with the fungus grown on nutrient plates. With increasing time, the number of upregulated unigenes increased, while the downregulated unigenes decreased. At 48 h, gene expressions of the biocontrol fungus were relative stable. Almost all the DEGs with maximum variations (either upregulated or downregulated) in different nutrient modes and growth stages were unknown genes or hypothetical genes with no specific functions reported.

The analysis of GO category and functional enrichment of the DEGs under sclerotial induction and vegetative growth showed the same enrichment terms in each category at 8 h, 24 h and 48 h, indicating an ongoing process of mycoparasitism; however, the proportions of specific functions differed among sampling times. The terms catalytic activity, cell and cell part and organic substance metabolic process were the most relevant and significant GO classifications of molecular function, cellular component and biological process, respectively.

By KEGG pathway assessment, all the DEGs were assigned to five types, with 108 KEGG pathways. The most frequently represented type was 'metabolic pathway', mainly including 'metabolic pathways', 'biosynthesis of secondary metabolites' and 'amino sugar and nucleotide sugar metabolism'. The second largest type was 'genetic information processing', mainly including 'RNA transport' and 'protein processing in endoplasmic reticulum'.

Figure 2. Clusters of orthologous groups (COG) functional classification of the unigenes of C. rosea 67-1. 8615 unigenes were functionally predicted and classified into 25 categories. Notations on the right represent the functions of the fungus.

Figure 3. Gene ontology (GO) categories of the unigenes of C. rosea 67-1. Three kinds of function, biological process, cellular components and molecular functions, are included in the GO analysis, and the percentage and number of the unigenes in each category are shown separately.
'Cell cycle-yeast' and 'meiosis-yeast' in the category of 'cellular process' also acted as typical pathways. However, the pathways of 'environmental information processing' with the typical pathway of 'MAPK signalling pathway' and 'organismal systems', represented by 'natural killer cell mediated cytotoxicity', had a quite low frequencies compared with the other types of pathways.

Quantitative real-time PCR verification. Twelve DEGs were selected and divided into two groups according to their expression levels. In the first group, containing four genes encoding an MFS transporter, aldehyde dehydrogenase, carbonic anhydrase and cysteine synthase, the gene expression levels increased successively throughout the process of sclerotial induction. In the other group, the DEGs were upregulated at 24 h but decreased at 48 h. The genes encoding endochitinase, monooxygenase, sugar transporter, N amino acid transport system protein, endoribonuclease, 4-aminobutyrate aminotransferase, hypothetical protein and alpha-galactosidase fitted this pattern (Fig. 5). The results of real-time PCR quantification were consistent with the DEGs analysis of the 67-1

| Pathway                                  | Proportion (%) | Level 1      | Level 2               |
|------------------------------------------|----------------|--------------|-----------------------|
| 1 Metabolic pathways                     | 31.6           | Metabolism   | Global map            |
| 2 Biosynthesis of secondary metabolites | 13.4           | Metabolism   | Global map            |
| 3 Starch and sucrose metabolism          | 8.9            | Metabolism   | Carbohydrate metabolism |
| 4 Amino sugar and nucleotide sugar metabolism | 5.0           | Metabolism   | Carbohydrate metabolism |
| 5 RNA transport                          | 4.1            | Genetic information processing | Translation |
| 6 MAPK signaling pathway                  | 4.1            | Environmental information processing | Signal transduction |
| 7 Purine metabolism                      | 3.4            | Metabolism   | Nucleotide metabolism |
| 8 Protein processing in endoplasmic reticulum | 3.1           | Genetic information processing | Folding, sorting and degradation |
| 9 Cell cycle                             | 2.9            | Cellular processes | Cell growth and death |
| 10 Spliceosome                           | 2.5            | Genetic information processing | Transcription |
| 11 Meiosis                               | 2.4            | Cellular processes | Cell growth and death |
| 12 RNA degradation                       | 2.4            | Genetic information processing | Folding, sorting and degradation |
| 13 Pyrimidine metabolism                 | 2.4            | Metabolism   | Nucleotide metabolism |
| 14 Ribosome                              | 2.1            | Genetic information processing | Translation |
| 15 Tyrosine metabolism                   | 2.1            | Metabolism   | Amino acid metabolism |
| 16 Lysine degradation                    | 2.1            | Metabolism   | Amino acid metabolism |
| 17 Peroxisome                            | 2.0            | Cellular processes | Transport and catabolism |

Table 2. KEGG analysis of the transcriptome of C. rosea 67-1. The KEGG Pathway analysis is divided into three levels. 1Percentage of unigenes in a pathway from among all the genes with KEGG pathway annotation (11595). Proportions lower than 2% are not listed.
transcriptome, indicating that the data obtained from transcriptome analysis were reliable for further analysing and selecting of parasitism-related genes in *C. rosea* during sclerotial induction.

**Discussion**

Understanding the mechanism of mycoparasitism of *C. rosea* would be of great value to improve biocontrol efficiency against plant fungal pathogens. In this study, a high-throughput sequencing and analysis of *C. rosea* induced by sclerotia of *S. sclerotiorum* was performed to explore mycoparasitism-related genes. The DEGs from the mycoparasite *T. harzianum* against fungal pathogens have been investigated by using suppression subtractive hybridization (SSH) method and RNA-seq technique in previous studies, in which autoclaved cell walls of *F. solani* and *S. sclerotiorum* were used as parasitic substrates compared with the saprophytic medium of glucose. However, the inactive cell walls of pathogenic fungi were more likely to provide a nutrient for vegetative growth of the mycoparasites, although certain genes would be turned on and expressed during the process of decomposing and utilizing the special substance supplemented, e.g. the fungal cell wall or chitin. In our research, fresh, non-autoclaved sclerotia of *S. sclerotiorum* were added and acted as an inducer and substrate, which represented the status of *C. rosea* parasitizing on *S. sclerotiorum*. In order to ensure no *S. sclerotiorum* mixing up with *C. rosea* samples, we determined the germination of sclerotia on agar before transcriptome sequencing and analysis, and found no hyphae extending within sampling time. We amplified and sequenced ITS in the samples as well, which arose a single peak for each base, indicating that all the samples came from *C. rosea* (Data not shown). Several

Figure 5. Verification of differentially expressed genes of *C. rosea* 67-1 under induction of sclerotia of *S. sclerotiorum* using quantitative real-time PCR. Relative expression levels of 12 unigenes (Cr1773, Cr13378, Cr12640, Cr13646, Cr8262, Cr5874, Cr10467, Cr8966, Cr5398, Cr2983, Cr13246 and Cr11906) from 67-1 during vegetative growth and during the mycoparasitic process are compared pairwise at different sampling times. The bars in blue with the small letter ‘a’ represent the control, and the bars in red with the letter ‘b’ represent treatment by adding fresh sclerotia. Error bars indicate the standard deviation of three replicates.
studies have suggested that when encountering with hyphae of pathogenic fungi, mycoparasites might display their biotrophic lifestyle\textsuperscript{6,27}. Using live sclerotia as the substrate, more DEGs and more reliable candidates associated with mycoparasitism of \textit{C. rosea} will be detected.

Generally, a series of activities are involved in the mycoparasitic process, including recognition, attachment, penetration and parasitism\textsuperscript{28}, and these behaviours of the antagonist are triggered in different stages of parasitism. In the co-culture of 67-1 and sclerotia, we found that the hyphae of 67-1 extended and attached to the sclerotia in 8 h. After 48 h the surface of the sclerotia was covered with hyphae of the mycoparasite and the mycelium of \textit{C. rosea} penetrating into internal tissues of the sclerotia was detected in the section under a microscope (Data not shown). Therefore, three time points (8 h, 24 h and 48 h), with and without sclerotia, were used to represent the whole mycoparasitic process of before penetration, mass invasion, and the later stage of mycoparasitism to construct the transcriptome of 67-1 parasitizing on sclerotia of \textit{S. sclerotiorum}. The results showed that 3,217 unigenes were upregulated and 2,739 were downregulated at 8 h. We deduced that upon the addition of sclerotia, the \textit{C. rosea} isolate changed its alimentation mode from saprophytic on PDA to parasitic, such that the fungus induced numerous genes related to parasitism and repressed many genes participating in vegetative growth on PDA medium. With increasing time, the upregulated unigenes increased successively compared with those expressed in the absence of sclerotia, indicating that the parasitic process of \textit{C. rosea} was ongoing. However, the number of downregulated unigenes decreased compared with that at 8 h. We hypothesized that the fungus turned off many genes involved in normal vegetative growth throughout the sampling times, resulting the observed decrease in downregulated unigenes at 24 and 48 h compared with the control. However, these speculations need further verified.

According to species distribution analysis, more than half of the unigenes annotated were parasitism-related, including both plant fungal pathogens, \textit{e.g.} \textit{F. solani} and \textit{F. oxysporum}, and entomogenous fungi \textit{Metarhizium} spp. and mycoparasites \textit{T. virens}, \textit{T. atroviride}, which show strong parasitic abilities to their hosts. Common genes discovered in different species may play critical roles in certain functions. Analysing the unigenes expressed in \textit{C. rosea} and detecting their expressions in other parasitic fungi represents an efficient and simple way to further identify and explore mycoparasitism-related genes.

Functional genes participating in the mycoparasitic process have received much attention in the last decades. Two genes encoding signal transduction proteins, G-protein and mitogen activated protein kinase (MAPK), were extensively studied and confirmed to be substantially involved in recognition and attachment of mycoparasites\textsuperscript{29–31}. From the transcriptome of \textit{C. rosea} 67-1, we also identified two unigenes encoding the G-protein alpha subunit and MAPK, respectively, whose expressions were remarkably upregulated under sclerotial induction compared with the control, indicating that both signal transduction-related proteins might be involved in regulating the activities of key enzymes in mycoparasitism or the formation of parasitic structures. Cell wall degrading enzymes are important factors in penetration and mycoparasitism\textsuperscript{32–34}. When 67-1 was induced by sclerotia, a series of cell wall degrading enzymes encoding genes, such as endochitinase, endoglucanase and serine protease, were remarkably upregulated. These hydrolases might play important roles in the interaction between mycoparasites and fungal pathogens by degrading cell wall, involving in penetration, and suppressing sporulation and germ tube extension of the pathogens. From the transcriptome, we also obtained several toxin encoding genes, which might effect the physiological activities of the pathogen and inhibit mycelial growth, leading to an increased mycoparasitic activity of \textit{C. rosea}.

As a mycoparasite, \textit{C. rosea} attacks its host actively and directly; however, it also possesses a resistance response to toxic metabolites secreted by \textit{S. sclerotiorum}. It was reported that ATP-binding cassette (ABC) transporter from \textit{C. rosea} was able to resist antifungal materials produced by pathogens. An ABC transporter-encoding gene, \textit{abcG5}, was isolated from the strain IK726 and functionally verified by gene deletion and complementation, which indicated that \textit{abcG5} was involved in cell protection by detoxifying \textit{Fusarium} mycotoxin zearealenone and other fungicides, thereby enhancing xenobiotic tolerance\textsuperscript{19,22}. In our study, the expression of a gene encoding an ABC transporter was induced in \textit{C. rosea} 67-1 in the presence of live sclerotia; thus, the toxin of \textit{S. sclerotiorum} might stimulate the resistance system of \textit{C. rosea}, and induce the expression of the ABC transporter-encoding gene to detoxify and protect \textit{C. rosea}.

Among the DEGs of 67-1, a number of functional unknown or hypothetical genes were identified. These genes were dramatically upregulated under sclerotial induction, some of them by more than 1,000-fold. These genes, especially the DEGs not expressed or rarely expressed in nutrient agar, but highly expressed under sclerotial induction, might be significant in mycoparasitism of \textit{C. rosea}. In the future, based on the information from the \textit{C. rosea} genome\textsuperscript{23} and the parasitic transcriptome, we will screen target genes and verify their specific functions in mycoparasitism by gene deletion, complementation and bioassay of \textit{S. sclerotiorum in vitro} and in the greenhouse.

Methods

Strains. \textit{C. rosea} 67-1 was originally isolated from a vegetable yard in Hainan Province, China. \textit{S. sclerotiorum} Ss-H was obtained from infected stems of soybeans in a field in Heilongjiang Province. Both strains are maintained in potato dextrose agar (PDA) in the Biocontrol of Soilborne Diseases Laboratory of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences.

Preparation of sclerotia. Agar blocks of Ss-H were inoculated into carrot medium\textsuperscript{36} and incubated at 26 °C. After 15 days, the sclerotia were harvested by rinsing with tap water to remove remnants of the medium. The sclerotia were air dried and stored in a cool place.

Preparation of mycelia samples of 67-1. The strain 67-1 was incubated on PDA plate at 26 °C for 10 days, and the spores were eluted by 5 ml sterile distilled water with a sterile glass spatula. The number of spores was counted under a microscope using a haemocytometer, and the concentration of the suspension was adjusted to 1 × 10^7 spores/ml. 100 μl of 67-1 suspension was dropped on the centre of a PDA plate (φ 90 mm) covered with a
85-mm sterile cellophane and smeared evenly with a sterile glass scraper. The fungus was cultured in an incubator at 26 °C for 48 h.

Uniformly sized sclerotia were picked up and surface-sterilized in 2.5% sodium hypochlorite solution for 3 min, and rinsed with sterile distilled water five times. Excess water remaining on the sclerotia was blotted off using sterile filter paper. The sclerotia were put onto the surface of 67-1 plates evenly to cover the whole colonies and co-cultured consecutively in an incubator at 26 °C. The sclerotia on the plates were took out using sterile forceps at the early stage (8 h), the intermediate stage (24 h) and the later period (48 h) of mycoparasitism, respectively, and the mycelia of 67-1 growing on the cellophane were collected using a sterile spatula. The 67-1 plates without added sclerotia acted as the control. The six samples of fungal hyphae were frozen immediately in liquid nitrogen and kept at −80 °C. Five replicates were conducted for each treatment, and the experiment was repeated for three times.

**Construction of cDNA libraries and sequencing.** The total RNAs of the mycelia samples of 67-1 were extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol, and DNase I (Invitrogen) was used to digest the genomic DNA completely. The concentration of total RNA was determined using an Ultraviolet Spectrophotometer (NanoDrop ND-1000, Wilmington, DE, USA) and its quality was tested using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the NanoDrop ND-1000 using the standards of 28S/18S > 1.0, OD260/230 ≥ 1.8, OD260/280 ≥ 1.8, and RNA Integrity Number > 6.5. 67-1 mRNA was extracted with Oligo (dT) magnetic beads and broken into small pieces using fragmentation buffer. Short cDNA fragments were synthesized using random hexamer primers, purified, resolved with EB buffer for end reparation and connected with adapters. Suitable fragments were selected as templates for PCR amplification. The quality of the six cDNA libraries was monitored with an Agilent 2100 Bioanalyzer and StepOnePlus Real-Time PCR System (ABI, CA, USA), and their sequences were determined using an Illumina HiSeq™ 2000 (Illumina Inc., CA, USA) in the Beijing Genome Institute (BGI).

**Bioinformatic analysis of 67-1 transcriptome sequences.** Raw reads of 67-1 cDNA libraries were filtered to remove adapter sequences, low quality reads and unknown nucleotides larger than 5%, and the clean reads obtained were submitted to the NCBI sequence read archive (SRA). De novo transcriptome assembly was carried out using the short reads assembly program Trinity37. The high quality reads were assembled into contigs, and then integrated to obtain unigenes. All the samples were derived from the same strain of C. rosea; therefore, the unigenes in each assembly could be further processed by sequence splicing and redundancy removal to obtain longer non-redundant unigenes.

Blastx alignment (E-value < 10^{-5}) was conducted to determine the sequence direction and to predict the protein coding regions. The CDSs were extracted from the unigenes, and those sequences that did not match any Blast results were predicted using the ESTScan program38. Annotation of the unigenes was performed using NR (Non-redundant), Swiss-Prot, GO (Gene ontology), KEGG (Kyoto encyclopedia of genes and genomes) and COG.
Analysis of DEGs. The expression levels of the 67-1 unigenes differentially expressed in different sampling times under sclerotial induction and on PDA medium were calculated by the fragments per kb per million fragments method (FPKM)\textsuperscript{42}. The false discovery rate (FDR) is considered as a standard to confirm the threshold of \( P \) value in multiple tests and analyses\textsuperscript{43}. An FDR < 0.001 and an absolute value ratio > 2 were adopted to judge the significance of gene expression differences between sclerotial induced and non-induced samples.

Quantitative real-time PCR certification. To verify the reliability of the transcriptome of \textit{C. rosea} 67-1, 12 genes that were upregulated in the DEG analysis were selected and quantified for their expression levels under sclerotial induction and during vegetative growth on PDA medium, using real-time PCR and internal reference gene \textit{Elongation factor 1} \textsuperscript{19}. Primer pair sets for the genes were designed using the software Primer Premier 6.0 (Table 3). The specificity of the primers was certified by conventional PCR with the following program: 94 °C for 3 min; 30 cycles of 94 °C for 1 min, 55 °C for 30 s and 72 °C for 30 s; followed by 72 °C for 10 min.

The total RNAs of the six samples were extracted and first strand cDNA was synthesized using a cDNA Synthesis Kit (Takara, Dalian, China). The expressions of the DEGs were assayed using a SYBR Premix Ex Taq (Takara) in an IQ \textcircled{S} \textsuperscript{TM} multicolor real-time PCR detection system (Bio-Rad, CA, USA) in a 25 μl reaction system containing 12.5 μl of SYBR Premix, 2 μl of 10-fold diluted cDNA, 1 μl of each primer and 8.5 μl of RNase-free water. The quantitative PCR was performed in a 96-well plate for 95 °C 2 min; 40 cycles of 95 °C 10 s and 55 °C 30 s. After the reaction, fluorescence values were collected every 0.5 °C from 55 °C to 85 °C for 81 cycles to check for non-specific amplification. The relative expression levels of the DEGs were calculated using the \( 2^{-\Delta\Delta C_{t}} \) method\textsuperscript{45}. Three replicates were performed for each template at each time point.

Statistical Analysis. The statistical software SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) was used for the analysis of variance (ANOVA) of the gene expression levels. Duncans multiple range test was used to compare the means of each treatment, and \( P \) value < 0.05 was considered significant.

References
1. Boland, G. I. & Hall, R. Index of plant hosts of \textit{Sclerotinia sclerotiorum}. \textit{Can. J. Plant Pathol.} \textbf{16}, 93–108 (1994).
2. Bardin, S. D. & Huang, H. C. Research on biology and control of Sclerotinia diseases in Canada. \textit{Can. J. Plant Pathol.} \textbf{23}, 88–98 (2001).
3. Hegedus, D. D. & Rimmer S. R. \textit{Sclerotinia sclerotiorum}: When "to be or not to be" a pathogen? \textit{FEMS Microbiol. Lett.} \textbf{251}, 177–184 (2005).
4. Papavizas, G. C. \textit{Trichoderma} and \textit{Gliocladium}: biology, ecology, and potential for biocontrol. \textit{Annu. Rev. Phytopathol.} \textbf{23}, 23–54 (1985).
5. Rodriguez, M. A., Cabrera, G., Gozzo, F. C., Eberlin, M. N. & Godeas, A. \textit{Clonostachys rosea} BAF3874 as a \textit{Sclerotinia sclerotiorum} antagonist: mechanisms involved and potential as a biocontrol agent. \textit{J. Appl. Microbiol.} \textbf{110}, 1177–1186 (2011).
6. Cota, L. V., Maffia, L. A., Mizubuti, E. S. G., Macedo, P. E. F. & Antunes, R. F. Biological control of strawberry gray mold by \textit{Clonostachys rosea} under field conditions. \textit{Biol. Control} \textbf{46}, 515–522 (2008).
7. Tu, J. C. \textit{Gliocladium virens} a destructive mycoparasite of \textit{Sclerotinia sclerotiorum}. \textit{Phytopathology} \textbf{70}, 674–678 (1980).
8. Dugan, F. M., Lupien, S. L., Hernandez-Bello, M., Peever, T. L. & Chen, W. Fungi resident in chickpea debris and their suppression of growth and reproduction of \textit{Didymella rabiei} under laboratory conditions. \textit{J. Phytopathol.} \textbf{153}, 431–439 (2005).
9. Harman, G. E., Howell, C. R., Viterbo, A., Chet, I. & Lorio, M. \textit{Trichoderma} species-opportunistic, avirulent plant symbionts. \textit{Nat. Rev. Microbiol.} \textbf{2}, 43–56 (2004).
10. Romão-Dumaresq, A. S., de Araujo, W. L., Talbot, N. J. & Thornton, C. R. RNA interference of endochitinases in the sugarcane endophyte \textit{Trichoderma virens} 223 reduces its fitness as a biocontrol agent of pineapple disease. \textit{Plos One} \textbf{7}, e47888 (2012).
11. Djonović, S., Pozo, M. I. & Kenerley, C. M. \textit{Tvbgn3}, a 3′-1.6-glucanase from the biocontrol fungus \textit{Trichoderma virens}, is involved in mycoparasitism and control of \textit{Pythium ultimum}. \textit{Appl. Environ. Microbiol.} \textbf{72}, 7661–7670 (2006).
12. Pozo, M. I., Baek, J. M., Garcia, J. M. & Kenerley, C. M. Functional analysis of \textit{tvsp1}, a serine protease-encoding gene in the biocontrol agent \textit{Trichoderma virens}. \textit{Fungal Genet. Biol.} \textbf{41}, 336–348 (2004).
13. Malmierca, M. G. et al. Involvement of \textit{Trichoderma} trichothecenes in the biocontrol activity and induction of plant defense-related genes. \textit{Appl. Environ. Microbiol.} \textbf{78}, 4856–4868 (2012).
14. Malmierca, M. G. et al. Relevance of trichothecenes in fungal physiology: Disruption of \textit{tris} in \textit{Trichoderma arundinaceum}. \textit{Fungal Genet. Biol.} \textbf{53}, 22–33 (2013).
15. Giczy, G., Kerényi, Z., Fülöp, L. & Horváth, L. Expression of \textit{cmg1}, an exo-beta-1,3-glucanase gene from \textit{Coniothyrium minitans}, increases during sclerotial parasitism. \textit{Appl. Environ. Microbiol.} \textbf{67}, 865–871 (2001).
16. Ren, L., Li, G. & Jiang, D. Characterization of some sugar factors affecting oxalate degradation by the mycoparasite \textit{Coniothyrium minitans}. \textit{J. Appl. Microbiol.} \textbf{108}, 173–180 (2010).
17. Zeng, F. X. et al. Fungal cell wall integrity-associated MAP kinase cascade in \textit{Coniothyrium minitans} is required for conidiation and mycoparasitism. \textit{Fungal Genet. Biol.} \textbf{49}, 347–357 (2012).
18. Hamid, M. I., Zeng, F. Y., Cheng, J. S., Jiang, D. H. & Fu, Y. Disruption of heat shock factor 1 reduces the formation of conidia and thermotolerance in the mycoparasitc fungus \textit{Coniothyrium minitans}. \textit{Fungal Genet. Biol.} \textbf{53}, 42–49 (2013).
19. Kosawang, C. et al. Zearalenone detoxification by zearalenone hydrolase is important for the antagonistic ability of \textit{Clonostachys rosea} against mycotoxigenic \textit{Fusarium graminearum}. \textit{Fungal Biol.} \textbf{118}, 364–373 (2014).
20. Mouekoubia, L. D. O., Zhang, L., Guan, X., Chen, X., Chen, H. & Zhang, J. Analysis of \textit{Clonostachys rosea}-induced resistance to tomato gray mold disease in tomato leaves. \textit{Plos One} \textbf{9}, e102690 (2014).
21. Zou, C. G. et al. Regulation of subtilisin-like protease \textit{prc} expression by nematode cuticle in the nematophagous fungus \textit{Clonostachys rosea}. \textit{Environ. Microbiol.} \textbf{12}, 3243–3252 (2010a).
22. Dubey, M., Jensen, D. F. & Karlsson, M. An ATP-binding cassette pleiotropic drug transporter protein is required for xenobiotic tolerance and antagonism in the fungal biocontrol agent \textit{Clonostachys rosea}. \textit{Mol. Plant Microbe Interact.} \textbf{27}, 725–732 (2014).
23. Zou, C. G., Tu, H. H., Liu, X. Y., Tao, N. & Zhang, K. Q. \textit{PacC} in the nematophagous fungus \textit{Clonostachys rosea} controls virulence to nematodes. \textit{Environ. Microbiol.} \textbf{12}, 1868–1877 (2010b).
24. Zhang, Y. H., Gao, H. L., Ma, G. Z. & Li, S. D. Mycoparasitism of \textit{Gliocladium roseum} 67-1 on \textit{Sclerotinia sclerotiorum}. \textit{Acta Phytopathol. Sin.} \textbf{34}, 211–214 (2004).
25. Vieira, P. M., Coelho, A. S. G., Steindorff, A. S., de Siqueira, S. J. L., Silva, R. & Ulhoa, C. J. Identification of differentially expressed genes from *Trichoderma harzianum* during growth on cell wall of *Fusarium solani* as a tool for biotechnological application. *BMC Genomics* **14**, 177 (2013).

26. Steindorff, A. S. *et al.* Identification of mycoparasitism-related genes against the phytopathogen *Sclerotinia sclerotiorum* through transcriptome and expression profile analysis in *Trichoderma harzianum*. *BMC Genomics* **15**, 204 (2014).

27. Chatterton, S. & Punja, Z. K. Chitinase and 1–3-β-glucanase enzyme production by the mycoparasite *Clonostachys rosea* f. *catenulate* against fungal plant pathogens. *Can. J. Microbiol.* **55**, 356–367 (2009).

28. Ye, X. B., Zeng, Q. C. & Jiang, X. L. Research advances on enzymes for mycoparasitism of *Trichoderma*. *Chin. J. Biol. Control* **25**, 276–280 (2009).

29. Reithner, B. *et al.* The G protein alpha subunit Tga1 of *Trichoderma atroviride* is involved in chitinase formation and differential production of antifungal metabolites. *Fungal Genet. Biol.* **42**, 749–760 (2005).

30. Zeilinger, S., Reithner, B., Scala, V., Peissl, I., Lorito, M. & Mach, R. Signal transduction by Tga3, a novel G protein alpha subunit of *Trichoderma atroviride*. *Appl. Environ. Microbiol.* **71**, 1591–1597 (2005).

31. Reithner, B., Schuhmacher, R., Stoppacher, N., Pucher, M., Brunner, K. & Zeilinger, S. Signaling via the *Trichoderma atroviride* mitogen-activated protein kinase Tmk 1 differentially affects mycoparasitism and plant protection. *Fungal Genet. Biol.* **44**, 1123–1133 (2007).

32. Geremia, R. A. *et al.* Molecular characterization of the proteinase encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. *Mol. Microbiol.* **8**, 603–613 (1993).

33. Carsolio, C., Gutierrez, A., Jimenez, B., Van Montagu, M. & Herrera-Estrella, A. Characterization of ech42, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 10903–10907 (1994).

34. Thrane, C., Tronsmo, A. & Jensen, D. F. Endo-1,3-β-glucanase and cellulase from *Trichoderma harzianum*; purification and partial characterization, induction of and biological activity against plant pathogenic *Pythium* spp. *Eur. J. Plant Pathol.* **103**, 331–344 (1997).

35. Sun, Z. B., Sun, M. H. & Li, S. D. Clone and functional analysis of endoglucanase gene from *Clonostachys rosea* strain 67-1. *Genome Announc.* **3**, e00354–15 (2015).

36. Zhang, Y., Sun, M. H., Li, S. D. & Luo, M. Cloning and functional analysis of endoglucanase gene from *Gliocladium catenulatum* HFL-1. *Chin. J. Biol. Control* **29**, 74–82 (2013).

37. Grabherr, M. G. *et al.* Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **29**, 644–652 (2011).

38. Isei, C., Jongeneel, C. V. & Bucher, P. ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences. *Proc. Int. Conf. Intell. Syst. Mol. Biol*. Heidelberg, Germany. California: AAAI Press. 138–148 (1999, August 6–10).

39. Conesa, A., Götz, S., Garcia-Gomez, J. M., Terol, J., Talon, M. & Robles, M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**, 3674–3676 (2005).

40. Tatusov, R. L. *et al.* The COG database: new developments in phylogenetic classification of proteins from complete genomes. *Nucleic Acids Res.* **29**, 22–28 (2001).

41. Clark, R. M. *et al.* Common sequence polymorphisms shaping genetic diversity in *Arabidopsis thaliana*. *Science* **317**, 338–342 (2007).

42. Audic, S. & Claverie, J. M. The significance of digital gene expression profiles. *Genome Res.* **7**, 986–995 (1997).

43. Benjamini, Y. & Yekutieli, D. The control of the false discovery rate in multiple testing under dependency. *Ann. Statist.* **29**, 1165–1188 (2001).

44. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCt method. *Methods* **25**, 402–408 (2001).

**Acknowledgements**

This research was supported by The National High Technology Research and Development Program (“863” Program) of China (2011AA10A205) and The National Key Technology R&D Program of China (2012BAD19B01).

**Author Contributions**

Z.S. conducted the experiment and analyzed the results. M.S. and S.L. conceived the experiment, analyzed the results and reviewed the manuscript.

**Additional Information**

**Accession code:** All the raw reads of six sets of 67-1 transcriptome were uploaded to the NCBI Short Read Archive (SRA) database with the accession numbers SRR1751506–1751511.

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Sun, Z. -B. *et al.* Identification of mycoparasitism-related genes in *Clonostachys rosea* 67-1 active against *Sclerotinia sclerotiorum*. *Sci. Rep.* **5**, 18169; doi: 10.1038/srep18169 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/