Screening and characterisation of proteins interacting with the mitogen-activated protein kinase Crmapk in the fungus Clonostachys chloroleuca

Binna Lv, Lele Fan, Shidong Li & Manhong Sun

Clonostachys chloroleuca 67-1 (formerly C. rosea 67-1) is a promising mycoparasite with great potential for controlling various plant fungal diseases. The mitogen-activated protein kinase (MAPK)-encoding gene Crmapk is of great importance to the mycoparasitism and biocontrol activities of C. chloroleuca. To investigate the molecular mechanisms underlying the role of Crmapk in mycoparasitism, a high-quality yeast two hybrid (Y2H) library of C. chloroleuca 67-1 was constructed, and proteins interacting with Crmapk were characterised. The library contained $1.6 \times 10^7$ independent clones with a recombination rate of 96%, and most inserted fragments were > 1 kb. The pGBKT7-Crmapk bait vector with no self-activation or toxicity to yeast cells was used to screen interacting proteins from the Y2H library, resulting in 60 candidates, many linked to metabolism, cellular processes and signal transduction. Combined bioinformatics and transcriptome analyses of C. chloroleuca 67-1 and ΔCrmapk mutant mycoparasitising Sclerotinia sclerotiorum sclerotia, 41 differentially expressed genes were identified, which might be the targets of the Fus3/Kss1-MAPK pathway. The results provide a profile of potential protein interactions associated with MAPK enzymes in mycoparasites, and are of great significance for understanding the mechanisms of Crmapk regulating C. chloroleuca mycoparasitism.
phosphorylate and activate downstream MAPKKs, which in turn phosphorylate and activate MAPKs, and these enzymes phosphorylate specific downstream substrates, initiating cellular responses\textsuperscript{17}. In \textit{S. cerevisiae}, five MAPK pathways that regulate the biological processes of mating (Fus3/Kss1), filamentous growth (Kss1), cell wall integrity (Slt2), responses to high osmotic stress (Hog1) and ascospore formation (Smk1)\textsuperscript{18,19} have been identified. The functions of MAPK-encoding genes have been studied in different species, including some biocontrol fungi in the last decade. Gruber and Zeilinger\textsuperscript{14} found that deletion of MAPK-encoding gene \textit{Tmk1} in \textit{Trichoderma atroviride} altered the radial growth and conidiation of the fungus, inhibited the formation of infection structures, and reduced mycoparasitic ability to \textit{R. solani} and \textit{B. cinerea} hyphae. In \textit{Coniothyrium minitans}, disruption of MAPK gene \textit{CmSlt2} resulted in a lack of conidiation and a marked reduction of mycoparasitic ability to the sclerotia of \textit{S. sclerotiorum}\textsuperscript{20}. However, MAPKs may also act as negative modulators in response to stimuli and/or stresses. Mendoza-Mendoza et al.\textsuperscript{21} reported that deficiency of MAPK gene \textit{Tvk1} induced the expression of mycoparasitism-related genes in \textit{T. virens} in response to \textit{R. solani}. In addition, the mutants exhibited enhanced biocontrol efficacy compared with the wild type strain and chemical fungicide.

During signal transmission, a series of proteins are activated responding to host and environmental stimulation. In plant pathogenic fungi, MAPKs interact with various kinds of proteins, such as transcription factor Ste12, MAPK phosphatase Msg5, and heat shock factor Sfl\textsuperscript{22–24}. However, there are no reports on proteins interacting with MAPK in biocontrol fungi, and the mechanism underlying MAPK-mediated regulation of mycoparasitism has not yet been clarified in mycoparasites.

Protein–protein interaction networks run through all biological and metabolic processes in living organisms. By using yeast two hybrid (Y2H) system, considerable interacting proteins and domains have been identified in plants, animals and fungi\textsuperscript{19,20,22}. Y2H technique was firstly developed in \textit{S. cerevisiae} by exploiting the transcription factor \textit{GAL4} containing a separable DNA binding domain (BD) and a transcriptional activation domain (AD)\textsuperscript{25}. Once prey proteins (library) fused to the DNA-AD domain interact with a bait protein (target) fused to the DNA-BD domain, \textit{GAL4} promoters are activated and nutritional or antibiotic selectable markers are expressed as reporter genes, revealing putative interacting proteins\textsuperscript{27}. Although some other methods such as coimmunoprecipitation and bimolecular fluorescence complementation are also employed\textsuperscript{28}, high-throughput screening of Y2H libraries remains the most cost-effective and practical approach for protein–protein interaction studies in vivo.

To explore Crmapk-interacting proteins in \textit{C. chloroleuca}, a high-quality Y2H library was generated using an in vivo recombination strategy, and 60 candidate interacting proteins involved in multiple biological processes were identified. To the best of our knowledge, this is the first report of proteins interacting with MAPK in \textit{C. chloroleuca} and other hyperparasites identified by constructing a Y2H library. The findings provide valuable clues to the mechanisms by which Crmapk regulates mycoparasitism of \textit{C. chloroleuca}.

**Results**

**Quality of Y2H library of \textit{C. chloroleuca} 67-1.** The mycelia of 67-1 strain under the induction of \textit{S. sclerotiorum} sclerotia were collected for RNA extraction and construction of Y2H library. The quality of the Y2H library was evaluated, revealing 1.6 × 10\textsuperscript{7} primary clones (Fig. 1a), much higher than the generally required capacity of 1.0 × 10\textsuperscript{6} CFU/mL. The recombination rate of the library was 96% (Fig. 1b), and 24 randomly selected
Colonies were found to have an average insert size of ~1 kb (Fig. 1c), indicating that the Y2H library was of high quality and could be used for protein–protein interaction analysis.

**Construction of the pGBK7-Crmapk bait vector and its auto-activation.** The domain serine/threonine protein kinase (1068 bp) of the Crmapk gene was amplified from cDNA of C. chloroleuca 67-1 using specific primers Crmapk-F/Crmapk-R (Fig. 2a). The pGBK7-Crmapk bait plasmid was successfully constructed and verified by PCR amplification using vector primers T7/3′BD and target gene primers Crmapk-F/Crmapk-R, and by DNA sequencing (Fig. 2b).

The availability of the pGBK7-Crmapk bait vector was determined, and colonies containing the bait plasmid were white in colour on SD/-Trp/X (synthetic dropout medium lacking tryptophan and supplemented with 40 µg/mL X-α-gal) plates, while colonies containing the pGADT7-T and pGBK7-53 positive control plasmids were blue, indicating that the pGBK7-Crmapk bait could not autonomously activate the reporter genes (Fig. 2c). In addition, from visual analysis, fungal colonies containing bait and control plasmids had apparently the same sizes, suggesting that the constructed vector was not toxic to yeast cells. Thus, the pGBK7-Crmapk bait vector could be used to screen the protein library for interacting proteins.

**Screening of Crmapk-interacting proteins.** After co-transformation of pGBK7-Crmapk and prey plasmids, a total of 288 blue clones grew on SD/-Leu/-Trp/X-α-Gal/AbA (DDO/X/A) plates (Fig. 3a), among which 149 remained blue on high-stringency SD/-Ade/-His/-Leu/-Trp/X-α-Gal/AbA (QDO/X/A) plates (Fig. 3b), indicating that these clones might be genuine positives. All prey plasmids were verified by PCR amplification using vector primers pGADT7-F/R. These positive clones were separately co-transformed with pGBK7-Crmapk, and finally, 80 blue clones emerged on QDO/X/A plates were obtained, which might express potential Crmapk-interacting proteins (Fig. 3c).

**Bioinformatic analysis of Crmapk-interacting proteins.** The sequences of interacting proteins were aligned with the C. chloroleuca 67-1 genome database, and 60 proteins interacting with Crmapk were identified (Supplementary Table S1). Among them, five genes presented higher interaction frequency than others, namely NODE_505_4, NODE_320_3, NODE_606_27, NODE_522_14 and NODE_689_14, encoding a mitochondrial distribution and morphology protein, a putative C2H2 zinc finger domain-containing protein, a phospho-2-dehydro-3-deoxyheptonate aldolase, a CCR4-NOT transcriptional complex subunit, and a glucose-6-phosphate 1-dehydrogenase, among which the interaction frequencies of NODE_505_4 and NODE_320_3 were 8 and 7, respectively. Additionally, several interacting proteins were vital components of signal transduction pathways, such as COP9 signalosome complex subunit 5 (NODE_52_12), ubiquitin-conjugating enzyme E2 (NODE_405_44), transport proteins SEC23 and SEC31 (NODE_374_8 and NODE_558_28), and translocation protein SEC66 (NODE_29_18), indicating that these proteins might be involved in the similar pathways with Crmapk. SMART and Pfam database analyses showed that the domains of the 60 interacting proteins were diverse and mainly included translation protein SH3-like domains, protein kinase domains, C2H2 finger domains, and other protein domains.
domains, peptidase S8/S53 domains, phosphofructokinase domains, and galactose mutarotase-like domains, implying that these domains might be involved in the interactions with Crmapk.

To investigate the categories of MAPK-interacting proteins in *C. chloroleuca*, the number of identified proteins related to each Gene Ontology (GO) term was calculated according to GO annotation information. The results for biological process classification showed that interacting proteins involved in cellular processes were the biggest group, accounting for 30% of all candidate proteins. The second largest group contained proteins associated with metabolic processes, accounting for 27% of identified proteins (Fig. 4a). In molecular function category, 37% of the candidates had the characters of binding ability, while 30% of proteins with catalytic and transporter activities might also play important roles during mycoparasitic processes in this biocontrol fungus (Fig. 4b). Subcellular localisation of the interacting proteins was also analysed, and they were mainly distributed in the nucleus (46%), cytoplasm (20%) and mitochondria (15%) of fungal cells (Fig. 4c). These results indicate that the putative MAPK-interacting proteins are involved in diverse functions during *C. chloroleuca* mycoparasitism, especially cellular, metabolism processes, and binding, catalytic activities.

**Protein–protein interaction verified by GST pull-down assay.** To further confirm the accuracy of the interactions identified by Y2H, six randomly selected putative interacting proteins NODE_320_3, NODE_439_51, NODE_505_4, NODE_1511_11, NODE_405_44 and NODE_606_27 that encoded a C2H2 zinc finger protein, an S8/S53 peptidase, a mitochondrial distribution and morphology protein, a NADH kinase...
pos5, a ubiquitin-conjugating enzyme E2, and a phospho-2-dehydro-3-deoxyheptonate aldolase, respectively, were assayed by the glutathione S-transferase (GST) pull-down method in vitro. The results showed that all the proteins could be eluted and were confirmed to interact with Crmapk (Fig. S1), indicating the Y2H results are reliable.

Expression of Crmapk-interacting genes in C. chloroleuca 67-1 under the induction of sclerotia. To investigate mycoparasitism-related genes in C. chloroleuca, the transcriptome of the strain 67-1 associated with S. sclerotiorum sclerotia was sequenced and the differentially expressed genes 8 h, 24 h and 48 h after sclerotia induction were analysed. Combining with these results, we found that 38 interacting genes were differentially expressed (|Log2 FC| ≥ 1 and \( p \leq 0.05 \); Table 1), among which 15 genes (NODE_15_2, NODE_17_1_30, NODE_176_9, NODE_198_20, NODE_2_38, NODE_201_16, NODE_374_8, NODE_38_86, NODE_486_20, NODE_507_12, NODE_52_12, NODE_525_9, NODE_558_28, NODE_606_27 and NODE_98_38) were upregulated by at least 1.2-fold at 8 h, similar to expression of Crmapk that especially highly expressed at 8 h during the mycoparasitic process of C. chloroleuca 67-1.

To explore the roles and mechanisms of Crmapk in C. chloroleuca mycoparasitism, the gene was deleted using gene homologous recombination strategy, and the transcriptome analysis of ΔCrmapk mutant mycoparasitising S. sclerotiorum sclerotia was performed before. In this study, it showed that when Crmapk was deficient, 11 putative interacting genes from the Y2H library were differentially expressed (|Log2 FC| ≥ 1 and \( p \leq 0.05 \)) based on the transcriptome profile of the C. chloroleuca mutant mycoparasitising sclerotia (Table 2), indicating that these genes might be involved in similar pathways to Crmapk. Among them, eight interacting genes were found to be differentially expressed in the wild type strain during parasitising sclerotia, including NODE_320_3, NODE_439_51, NODE_29_18 and NODE_17_25 encoding a C2H2 zinc finger protein, an S8/S53 peptidase, translocation protein SEC66, and N-acetyl-beta-D-glucosaminidase (NAGase), suggesting that they might account for the decreased mycoparasitic ability of C. chloroleuca 67-1. From above results, we speculate that all the differentially expressed genes maybe the targets of the Fus3/Kss1-MAPK pathway.

In order to verify whether the transcriptome profiles were reliable for screening Crmapk-interacting proteins in C. chloroleuca, the relative expressions of nine differently expressed genes during mycoparasitising were analysed by using quantitative reverse transcription PCR (qRT-PCR), and the detailed information was shown in Supplementary Table S1. The results confirmed that these Crmapk-interacting genes encoding acetyl-CoA carboxylase, DNA polymerase eta subunit, trihydroxynaphthalene reductase, transport protein SEC23, aflatoxin B1 aldehyde reductase, glucoamylase, COP9 signalosome complex subunit 5, asparagine synthetase domain-containing protein, and phospho-2-dehydro-3-deoxyheptonate aldolase were consistent with the transcriptome data of C. chloroleuca 67-1 (Fig. 5). This suggests that the transcriptome profiles are reliable, and suitable for screening Crmapk-interacting proteins and probing the mechanisms by which Crmapk regulates mycoparasitism of C. chloroleuca.
Discussion

MAPK signal transduction pathways are highly conserved in eukaryotic cells, and can modulate multiple cellular processes including embryogenesis, proliferation, differentiation, virulence and hyphal chemotropism through signal transduction from the surface of cells stimulated by extracellular factors. In a previous study, it was confirmed that Crmapk played an important role in mycoparasitism of C. chloroleuca. Crmapk is a protein kinase that acts as the central regulator of signal transduction pathways in C. chloroleuca, but its regulatory mechanisms are unknown. Proteins often work together with other protein partners to accomplish their essential functions in living organisms. Therefore, characterisation of proteins interacting with Crmapk may provide information for understanding the molecular mechanisms by which Crmapk regulates mycoparasitism of C. chloroleuca. In previous studies, several MAPK-interacting proteins have been identified, including transcription factors Mst12 and Mcm1, and dual-specificity protein kinase Msg5 in pathogenic fungi. In the present study, we constructed a high-quality Y2H library using the hyphae of C. chloroleuca induced by the sclerotia of S. sclerotiorum, from

| Gene ID      | Log2 FC (B8/A8) | Log2 FC (B24/A24) | Log2 FC (B48/A48) | Annotation                                      |
|--------------|-----------------|-------------------|-------------------|------------------------------------------------|
| NODE_115_3   | 0.31            | 1.84              | 1.10              | Acetyl-CoA carboxylase                           |
| NODE_130_53  | 0.55            | 0.25              | 1.04              | 6-Phosphofructokinase subunit alpha             |
| NODE_1331_7  | –0.66           | 0.90              | 3.52              | DNA polymerase eta subunit                       |
| NODE_15_2    | 2.33            | 1.60              | 2.67              | Glucoamylase                                    |
| NODE_1511_11 | –1.19           | –1.52             | –2.39             | NADH kinase pos5                                |
| NODE_1511_79 | –0.38           | 0.41              | 1.08              | Methionine aminopeptidase                        |
| NODE_17_25   | –0.14           | –0.38             | 1.87              | N-acetyl-beta-D-glucosaminidase                  |
| NODE_171_30  | 1.38            | 1.42              | 1.52              | Alpha-mannosidase                               |
| NODE_176_9   | 1.62            | 1.36              | 2.26              | Protein transport protein sec-13                |
| NODE_19_9    | –2.18           | –1.40             | –0.51             | Hypothetical protein                            |
| NODE_198_20  | 1.79            | 0.80              | 0.39              | SH3 domain-containing protein                   |
| NODE_2_38    | 1.22            | 0.53              | 0.31              | Glutamate decarboxylase                         |
| NODE_201_16  | 1.78            | 2.15              | 1.93              | Trihydroxyoxiniflumel reductase                 |
| NODE_228_20  | –0.76           | –0.57             | 1.22              | Aldose 1-epimerase                              |
| NODE_2303_57 | 0.54            | 0.46              | 2.07              | Flocculation protein FLO11                      |
| NODE_254_7   | –0.78           | –1.31             | –0.31             | Serine/threonine-protein kinase srk1            |
| NODE_281_31  | –1.59           | –0.49             | 0.09              | Ribosome biogenesis protein NSA2                |
| NODE_29_18   | 0.76            | 0.93              | 1.25              | Translocation protein sec6                      |
| NODE_320_3   | –0.44           | –0.15             | –2.64             | Putative C2H1 finger domain-containing protein   |
| NODE_374_8   | 3.02            | 2.63              | 2.22              | Transport protein SEC23                         |
| NODE_38_86   | 1.78            | 2.27              | –0.11             | Aflatoxin B1 aldehyde reductase member 2        |
| NODE_403_52  | –0.53           | –1.18             | –0.57             | Zinc finger protein ADR1                        |
| NODE_408_35  | –0.52           | –0.23             | 1.10              | Nucleoporin nsp1                                |
| NODE_439_51  | –1.10           | 1.52              | –1.52             | Peptidase S8 and S53                            |
| NODE_486_20  | 3.33            | 3.51              | 3.30              | Glucoamylase                                    |
| NODE_492_8   | –1.45           | –1.56             | –0.78             | Hypothetical protein                            |
| NODE_505_4   | –1.92           | –3.24             | –0.36             | Mitochondrial distribution and morphology protein|
| NODE_507_12  | 1.91            | 1.55              | 1.96              | Kinesin heavy chain                             |
| NODE_514_22  | –1.08           | –0.28             | –1.07             | C2H1 type zinc finger domain-containing protein  |
| NODE_52_12   | 2.50            | 2.06              | 1.34              | COP9 signalsome complex subunit 5               |
| NODE_525_16  | 0.73            | –0.26             | 1.12              | DNA replication regulator SLD3                  |
| NODE_525_9   | 1.36            | 1.63              | 1.75              | Asparagine synthase-like protein                |
| NODE_558_28  | 1.48            | 0.26              | 2.53              | Protein transport protein SEC31                 |
| NODE_606_27  | 2.19            | 1.61              | 1.18              | Phospho-2-dehydro-3-deoxyheptonate aldolase     |
| NODE_69_11   | –2.90           | –1.11             | 2.29              | Glucosamine 6-phosphate deaminase               |
| NODE_98_38   | 2.30            | 2.13              | 1.02              | Elongation factor 2                             |
| NODE_98_59   | –1.10           | 1.53              | 1.87              | Splicing factor 1                               |
| NODE_98_63   | 0.74            | 1.08              | 0.74              | DENN domain-containing protein                  |

Table 1. Genes interacting with Crmapk that are differentially expressed in the transcriptome of C. chloroleuca 67-1 parasitising S. sclerotiorum. Log2 FC, the abbreviation of Log [base 2] fold change, represents the relative expression of each gene (log2 value) between the control and treatments. The letter ‘A’ represents the controls (samples without sclerotia) and “B” represents the treatments induced by S. sclerotiorum sclerotia. The samples of two groups were collected at 8 and 24 h, respectively, and the data of gene expressions were derived from our previous research.
positive interactions. Although in some cases, Y2H analyses may generate a number of false-positives, the

samples of two groups were collected at 8 and 24 h, respectively, and the data of gene expressions were derived

S. sclerotiorum sclerotia. The relative expression of each gene (log2 value) between the control and treatments. The letter “A” represents the

signalosome may play an important role in signal transduction. Proteins may act as positive or negative regulators in MAPK signalling pathways to medi-

frequency. The C2H2 zinc finger family is a large group of transcription factors that play important roles in a

complex, transcription factors and translocators, which involved in gene regulation, metabolism and signal

are new and perform diverse functions in filamentous fungi. Many of these proteins are related to signalosome

transmission. In the present study, 60 proteins putatively interacting with Crmapk were identified, most of which

C. chloroleuca parasitism of S. sclerotiorum. The mutant parasitising S. sclerotiorum was differentially expressed, suggesting that it may contribute to the mycoparasitic ability of C. chloroleuca.

In many biological processes in eukaryotic organisms, MAPKs interact with other proteins to facilitate signal transmission. In the present study, 60 proteins putatively interacting with Crmapk were identified, most of which act new and perform diverse functions in filamentous fungi. Many of these proteins are related to signalosome complex, transcription factors and translocators, which involved in gene regulation, metabolism and signal transduction processes. Among them, C2H2-type zinc finger protein NODE_320_3 displayed high screening frequency. The C2H2 zinc finger family is a large group of transcription factors that play important roles in a variety of cellular functions, including gene expression, cell growth, proliferation, apoptosis and intracellular signal transduction. Proteins may act as positive or negative regulators in MAPK signalling pathways to mediate cellular functions. From the transcriptome of strain 67-1 under the induction of sclerotia, we found that NODE_320_3 was differentially expressed, suggesting that it may contribute to the mycoparasitic ability of C. chloroleuca.

NODE_52_12 functions as a COP9 signalosome (CSN) complex. The CSN/COP9 signalosome has been

shown to act in many pathways, mainly via ubiquitin degradation and signal transduction pathways. Brock-

way et al. demonstrated that deficiency of the csn gene resulted in an inability to activate the MAPK pathway,

which interrupted proliferation of Caenorhabditis elegans. In the present study, the CSN gene was also found to be markedly upregulated during the mycoparasitic process of C. chloroleuca 67-1, indicating that the COP9 signalosome may play an important role in C. chloroleuca biocontrol activities.

Interacting proteins are more likely to be involved in similar biological processes and functions, and may

do not identify putative interactions between two functional proteins, but also has great potential for high-throughput screening of uncharacterised proteins that bind to a given bait protein in large pooled cDNA libraries. In the current study, we harvested mycelia from C. chloroleuca associated with S. sclerotiorum sclerotia rather than collecting samples from culture media, and thereby constructed a mycoparasitism-related cDNA library. Undoubtedly, a high-quality cDNA library is of great importance for obtaining reliable interaction data in Y2H analyses. In this study, the library capacity, recombination rate and size of inserted fragments that are important to the quality of cDNA libraries fulfilled the requirements and ensured the specificity and accuracy of the positive interactions. Although in some cases, Y2H analyses may generate a number of false-positives, the proteins identified herein are highly likely to be related to the mechanism by which Crmapk regulates myco-

parasitism of C. chloroleuca.

| Gene ID      | Log, FC(B8/A8) | Log, FC(B24/A24) | Annotation                               |
|-------------|----------------|------------------|-----------------------------------------|
| NODE_1511_11| 1.74           | 1.58             | NADH kinase pos5                         |
| NODE_17_25  | 3.18           | 1.59             | N-acetyl-beta-D-glucosaminidase          |
| NODE_19_9   | -1.48          | -2.55            | Hypothetical protein                     |
| NODE_2303_85| -1.38          | -1.00            | Golgi apyrase                            |
| NODE_29_18  | -1.18          | -0.06            | Translocation protein sec66              |
| NODE_320_3  | -4.70          | -4.29            | Putative C2H2 finger domain-containing protein |
| NODE_439_51 | -0.94          | -2.57            | Peptidase S8 and S53                     |
| NODE_492_8  | -2.93          | -3.00            | Hypothetical protein                     |
| NODE_505_4  | -1.32          | -1.84            | Mitochondrial distribution and morphology protein |
| NODE_99_2   | 0.64           | 1.03             | DNA repair protein rad14                 |
| NODE_990_5  | -1.83          | -1.76            | Hypothetical protein                     |

Table 2. Genes interacting with Crmapk that are differentially expressed in the transcriptome of the ΔCrmapk mutant parasitising S. sclerotiorum. Log, FC, the abbreviation of Log [base 2] fold change, represents the relative expression of each gene (log2 value) between the control and treatments. The letter “A” represents the controls (samples without sclerotia) and “B” represents the treatments induced by S. sclerotiorum sclerotia. The samples of two groups were collected at 8 and 24 h, respectively, and the data of gene expressions were derived from our previous research.

which proteins potentially interacting with Crmapk were screened and identified. To the best of our knowledge, this is the first report of identification of proteins interacting with MAPK in mycoparasites.

The Y2H system is widely used for the discovery of protein–protein interactions. This approach not only identifies putative interactions between two functional proteins, but also has great potential for high-throughput screening of uncharacterised proteins that bind to a given bait protein in large pooled cDNA libraries. In the current study, we harvested mycelia from C. chloroleuca associated with S. sclerotiorum sclerotia rather than collecting samples from culture media, and thereby constructed a mycoparasitism-related cDNA library. Undoubtedly, a high-quality cDNA library is of great importance for obtaining reliable interaction data in Y2H analyses. In this study, the library capacity, recombination rate and size of inserted fragments that are important to the quality of cDNA libraries fulfilled the requirements and ensured the specificity and accuracy of the positive interactions. Although in some cases, Y2H analyses may generate a number of false-positives, the proteins identified herein are highly likely to be related to the mechanism by which Crmapk regulates mycoparasitism of C. chloroleuca.

In many biological processes in eukaryotic organisms, MAPKs interact with other proteins to facilitate signal transmission. In the present study, 60 proteins putatively interacting with Crmapk were identified, most of which act new and perform diverse functions in filamentous fungi. Many of these proteins are related to signalosome complex, transcription factors and translocators, which involved in gene regulation, metabolism and signal transduction processes. Among them, C2H2-type zinc finger protein NODE_320_3 displayed high screening frequency. The C2H2 zinc finger family is a large group of transcription factors that play important roles in a variety of cellular functions, including gene expression, cell growth, proliferation, apoptosis and intracellular signal transduction. Proteins may act as positive or negative regulators in MAPK signalling pathways to mediate cellular functions. From the transcriptome of strain 67-1 under the induction of sclerotia, we found that NODE_320_3 was differentially expressed, suggesting that it may contribute to the mycoparasitic ability of C. chloroleuca.

NODE_52_12 functions as a COP9 signalosome (CSN) complex. The CSN/COP9 signalosome has been shown to act in many pathways, mainly via ubiquitin degradation and signal transduction pathways. Brockway et al. demonstrated that deficiency of the csn gene resulted in an inability to activate the MAPK pathway, which interrupted proliferation of Caenorhabditis elegans. In the present study, the CSN gene was also found to be markedly upregulated during the mycoparasitic process of C. chloroleuca 67-1, indicating that the COP9 signalosome may play an important role in C. chloroleuca biocontrol activities.

Interacting proteins are more likely to be involved in similar biological processes and functions, and may be co-expressed in some circumstances. In the transcriptome of C. chloroleuca 67-1 during the mycoparasitic process, Crmapk was especially highly expressed at 8 h, indicating its involvement in initiating C. chloroleuca mycoparasitism. Combined with transcriptome analyses, the results showed that 15 interacting genes were upregulated during the early stages of the mycoparasitic process, consistent with the Crmapk expression profile. We speculate that these genes are likely to be closely connected with Crmapk, and involved in signal transduction following pathogen stimulation.

Peptidases of the S8/S53 family can degrade a broad range of substrates, including plant nematodes, and one such protein has been linked to the molecular mechanism of C. rosea infection of nematodes. Pozo et al. found that overexpression of the serine protease encoding gene tsp1 in T. virens significantly increased its ability to protect cotton seedlings against R. solani, and the gene was involved in the biocontrol process of the fungus. More interestingly, the S8/S53 protease gene family is in fact evolving under selection for increased gene copy number in C. rosea, which highlights its importance for mycoparasitism. Herein, we found that peptidase S8/S53 gene
NODE_439_51 was differentially expressed in both wild-type and ΔCrmapk mutant strains during parasitism on S. sclerotiorum, implying that this peptidase might be involved in C. chloroleuca infection of pathogenic fungi.

Chitinolytic enzymes which are essential for catabolism of chitin primarily include chitinase, NAGase, and lytic polysaccharide monooxygenase. Among them, NAGase that belongs to GH family 20 is capable of hydrolysing the terminal N-acetylglucosamine residues and exhibits a variety of biological activities. The previous study of T. harzianum proved that the expression of the NAGase gene could be activated by three kinds of chitin-rich residues, chitosan, shrimp shell powder and mushroom wastes, sequentially enhanced the chitinolytic activity, which facilitated the biocontrol efficacy of T. harzianum against F. oxysporum in greenhouse. In the present study, we found that NAGase gene NODE_17_25 was differentially expressed both in the wild-type and ΔCrmapk mutant strains parasitising S. sclerotiorum, providing us a new insight into the biocontrol mechanism of C. chloroleuca.

Another interesting gene is NODE_29_18 that encodes a translocation protein. Translocation proteins play auxiliary roles in recognition of precursors, and are closely related to signal transduction pathways. In the

Figure 5. Expression levels of nine Crmapk-interacting genes in C. chloroleuca 67-1 under mycoparasitic conditions. The mycelia of 67-1 strain under the induction of sclerotia were collected at 8, 24 and 48 h. The letter “a” represents the controls (samples without sclerotia) and “b” represents the treatments induced by S. sclerotiorum sclerotia. Error bars indicate the standard deviation of three replicates. Statistical tests were carried out using Tukey’s test for multiple comparisons. Asterisks indicate statistically significant difference (p < 0.05).
present study, we found that this gene was differentially expressed in both isolates, indicating that the translocation protein may be closely related to MAPK pathways and involved in the mycoparasitic process of *C. chloroleuca*.

Based on the above findings, a regulatory network of *C. chloroleuca* mycoparasitism could be constructed from Crmapk and its interacting proteins. We speculate that once encountering a fungal host, Crmapk is highly expressed in *C. chloroleuca*, and simultaneously stimulates a series of related pathways to complete signal transduction and initiate the mycoparasitic process.

In conclusion, we identified putative MAPK-interacting proteins in *C. chloroleuca*, and the results may be applicable to other mycoparasites. The findings provide vital clues regarding molecular mechanisms by which Crmapk regulates mycoparasitism of *C. chloroleuca*, improve our knowledge of the mechanisms underlying biocontrol of *C. chloroleuca*, and help to the development of highly efficient biocontrol agents.

**Materials and methods**

**Fungal strains and plasmids.** *C. chloroleuca* 67-1 (ACCC 39160) was originally isolated from a vegetable yard in Hainan Province, China, using the sclerotia-baiting method. *S. sclerotiorum* SS-H (ACCC 39161) was separated from sclerotia-infected soybean stems in a field in Heilongjiang Province, China. Both strains were regularly cultured on potato dextrose agar (PDA) medium at 26 °C and maintained at 4 °C in the Biocontrol of Soilborne Diseases Lab of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences.

**Construction of the Y2H library of *C. chloroleuca* 67-1.** Strain 67-1 was incubated on PDA at 26 °C for 10 days, spores were washed with sterile water and adjusted to 1 × 10^{7} spores/mL, and spore suspensions were smeared evenly on a PDA plate and covered with cellophane. Uniformly sized sclerotia were placed onto the surface of strain 67-1 plates evenly after culturing for 48 h, and the mycelia of *C. chloroleuca* 67-1 during mycoparasitic process and vegetative growth were collected, respectively, at 24 h and placed immediately in liquid nitrogen. Each treatment included five replicates.

Total RNA was harvested using TRIzol reagent (Invitrogen, California, USA), then treated with DNase I (TransGen, Beijing, China) to eliminate contaminated genomic DNA. The purity and integrity of total RNA were determined using a NanoDrop 1000 instrument (Thermo, Wallham, USA) and agarose gel electrophoresis, respectively. The isolation and purification of poly(A) mRNA from total RNA were carried out using an Oligotex mRNA Midi Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The cDNA library was acquired using a CloneMiner II cDNA Library Construction Kit (Invitrogen) according to the manufacturer’s protocols.

Following normalisation and short fragment removal, the purified cDNA and linearised pGADT7-DEST vector (prey plasmid), including three different reading frames to confirm the correct expression of all proteins, were co-transformed into the Y187 yeast strain using Yeastmaker Yeast Transformation System 2 (Clonetech, Shiga, Japan). After culturing on synthetic defined medium lacking leucine (SD/-Leu) to select transformants, a series of dilutions of the transformed mixture were also spread on SD/Leu plates to calculate the transformation efficiency and isolate separate colonies. After culturing at 30 °C for 3–6 days, positive transformants were harvested to form an Y2H library. A hemacytometer (Thermo, Wallham, USA) was used to measure the cell density of the Y2H library to ensure that the library capacity was no less than 1.0 × 10^{9} CFU, which is essential for a high-quality Y2H library. To check insert sizes and the recombination rate of the Y2H library, 24 colonies were randomly picked out and amplified by PCR using primers pGADT7-F/R (Table 3). The primary library was retransformed into competent *Escherichia coli* DH10B, and the plasmids were harvested and stored at −80 °C.

**Construction of the pGBK7-Crmapk bait vector.** To screen interacting proteins from the *C. chloroleuca* Y2H library, the pGBK7-Crmapk bait vector was constructed. Using *C. chloroleuca* cDNA as a template, the full-length coding sequence (CDS) of Crmapk (GenBank accession number: KY701731) was amplified with specific primers Crmapk-F and Crmapk-R (Table 3), containing restriction sites BamHI and EcoRI. The resulting PCR product was purified and inserted into bait vector pGBK7 harbouring the GAL4 DNA-binding domain (BD). The recombinant pGBK7-Crmapk bait plasmid was verified by double restriction enzyme digestion and DNA sequencing (TSINGKE, Beijing, China).

**Auto-activation tests of the pGBK7-Crmapk bait vector.** Bait vector pGBK7-Crmapk and empty vector pGADT7 were separately transformed into the Y2H Gold strain using the PEG/LiAc-mediated method. The vectors pGBK7-T53 and pGADT7-T co-transformed were used as positive control, while pGBK7-Lam and pGADT7-T were used as negative control. Transformants were grown on SD/-Trp (SD medium lacking tryptophan), SD/-Trp/X and SD/-Trp/X/A (SD- Trp supplemented with 40 µg/mL X-a-gal and 200 ng/mL aureobasidin A) plates at 30 °C for 3–5 days. Following the growth of white colonies on SD/-Trp and SD/-Trp/X plates, and no colony growth on SD/-Trp/X/A plates, the bait was verified without auto-activation. In addition, if the bait was toxic, the colonies containing the bait plasmid were obviously smaller than the control treatments. Only the bait vectors that showed no auto-activation activity or toxicity were used for Y2H screening.

**Screening of Crmapk-interacting proteins.** In order to screen proteins interacted with Crmapk, bait vector pGBK7-Crmapk and the Y2H AD library were co-transformed into the Y2H Gold strain using the yeast mating method. The mating culture was plated on DDO/X/A agar plates at 30 °C for 3–5 days, and all blue colonies on DDO/X/A agar plates were patched onto higher stringency QDO/X/A agar plates. To further verify the interactions, the prey plasmids of each primary interacting protein were rescued from yeast strains and separately co-transformed with pGBK7-Crmapk into the Y2H Gold yeast cells. The transformants were

https://doi.org/10.1038/s41598-022-13899-3
then retested on DDO/X/A and QDO/X/A media at 30 °C for 3–5 days, after which resulting blue colonies were considered to be potential positive clones. At the same time, the pGBKT7-53 and pGADT7-T plasmids were co-transformed into Y2H Gold cells as a positive control, while the pGBKT7-Lam and pGADT7-T served as a negative control. Three replicates were carried out, and all positive clones were identified by DNA sequencing using primers pGADT7-F/R for the T7 promoter.

**GST pull-down assay.** The DNA fragment of Crmapk was cloned into the vector pGEX-4T-1 (GE Healthcare, Chicago, United States) to generate the Crmapk-GST fusion protein. Six interacting proteins NODE_320_3, NODE_439_51, NODE_505_4, NODE_1511_11, NODE_405_44 and NODE_606_27 localising in the nucleus, cytoplasm and mitochondria basing on bioinformatics analyses were selected randomly and corresponding his-labelled proteins were constructed in the pCZN1 (Zoonbio, Nanjing, China) vector separately. GST, Crmapk-GST, NODE_320_3-his, NODE_439_51-his, NODE_505_4-his, NODE_1511_11-his, NODE_405_44-his and NODE_606_27-his plasmids were expressed in the E. coli BL21 cells (Sangon, Shanghai, China). The cells were lysed in lysis buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM PMSF) with a sonicator (Scientz, Ningbo, China) and centrifuged at 13,000 g, for 10 min. The supernatants were transferred to a 1.5 mL tube and stored at −70 °C. The GST and Crmapk-GST supernatants were mixed with 30 μl glutathione Sepharose beads (GE Healthcare) and incubated at 4 °C for 2 h, and then the recombinants of Crmapk-GST and GST bound to the Sepharose beads were incubated with the cell lysate of E. coli that contained NODE_320_3-his, NODE_439_51-his, NODE_505_4-his, NODE_1511_11-his, NODE_405_44-his and NODE_606_27-his plasmids were expressed in the E. coli BL21 cells (Sangon, Shanghai, China). The cells were lysed in lysis buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM PMSF) with a sonicator (Scientz, Ningbo, China) and centrifuged at 13,000g for 10 min. The supernatants were transferred to a 1.5 mL tube and stored at −70 °C. The GST and Crmapk-GST supernatants were mixed with 30 μl glutathione Sepharose beads (GE Healthcare) and incubated at 4 °C for 2 h, and then the recombinants of Crmapk-GST and GST bound to the Sepharose beads were incubated with the cell lysate of E. coli that contained NODE_320_3-his, NODE_439_51-his, NODE_505_4-his, NODE_1511_11-his, NODE_405_44-his and NODE_606_27-his plasmids were expressed in the E. coli BL21 cells (Sangon, Shanghai, China). The cells were lysed in lysis buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM PMSF) with a sonicator (Scientz, Ningbo, China) and centrifuged at 13,000g for 10 min. The supernatants were transferred to a 1.5 mL tube and stored at −70 °C. The GST and Crmapk-GST supernatants were mixed with 30 μl glutathione Sepharose beads (GE Healthcare) and incubated at 4 °C for 2 h, and then the recombinants of Crmapk-GST and GST bound to the Sepharose beads were incubated with the cell lysate of E. coli that contained NODE_320_3-his, NODE_439_51-his, NODE_505_4-his, NODE_1511_11-his, NODE_405_44-his and NODE_606_27-his plasmids were expressed in the E. coli BL21 cells (Sangon, Shanghai, China). The cells were lysed in lysis buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM PMSF) with a sonicator (Scientz, Ningbo, China) and centrifuged at 13,000g for 10 min. The supernatants were transferred to a 1.5 mL tube and stored at −70 °C. The GST and Crmapk-GST supernatants were mixed with 30 μl glutathione Sepharose beads (GE Healthcare) and incubated at 4 °C for 2 h, and then the recombinants of Crmapk-GST and GST bound to the Sepharose beads were incubated with the cell lysate of E. coli that contained NODE_320_3-his, NODE_439_51-his, NODE_505_4-his, NODE_1511_11-his, NODE_405_44-his and NODE_606_27-his plasmids were expressed in the E. coli BL21 cells (Sangon, Shanghai, China).

**Bioinformatics analyses of Crmapk-interacting proteins.** To identify the corresponding interacting proteins, all sequences obtained from the Y2H assays were analysed using BLAST (https://blast.ncbi.nlm.nih.gov). Homologous proteins were also identified in S. cerevisiae by BLAST. Next, GO analysis (http://amigo.geneontology.org/amigo) was performed to probe gene functional classification basing on biological process, molecular function and subcellular localisation. KEGG pathway analysis (https://www.kegg.jp/) was conducted to investigate high-level functions and pathways from molecular-level information. Protein function and interaction networks of the identified interacting proteins were generated using the UniProt database (http://www.uniprot.org).
qRT-PCR detection of Crmapk-interacting genes. Nine differentially expressed interacting genes (NODE_115_3, NODE_1331_7, NODE_201_16, NODE_374_8, NODE_38_86, NODE_486_20, NODE_52_12, NODE_525_9, NODE_606_27) were selected randomly and their expression levels during mycoparasitism on S. sclerotiorum sclerotia were determined by qRT-PCR as previously described. The letter 'a' represents the controls (samples without sclerotia) and 'b' represents the treatments by S. sclerotiorum sclerotia. The mycelia of C. chlороleuca 67-1 under the induction of sclerotia were collected at 8, 24 and 48 h, and total RNA was extracted using TRIzol reagent following the manufacturer's instructions. The cDNA was prepared from total RNA using a cDNA FastQuant RT Kit (TIANGEN, Beijing, China), and the expression of Crmapk-interacting genes derived from the Y2H library was investigated using a Bio-Rad IQ 5 Real-Time System (Bio-Rad, California, USA) and SYBR Premix Ex Taq (Takara, Dalian, China). Elongation factor gene EF1 (GenBank accession number: KP274074) was used as an internal reference to normalise gene expression, and the primers used for qRT-PCR determination of the candidates were listed in Table 3. Amplification by qRT-PCR involved heating at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 55 °C for 30 s. After PCR amplification, fluorescence values were measured every 0.5 °C from 55 to 95 °C, and the relative expression levels of Crmapk-interacting genes were calculated using the 2−∆∆Ct method. All reactions were performed in triplicate. Statistical software SPSS 2.0 (Chicago, IL, USA) was used for ANOVA. Statistical tests were carried out using Tukey's test for multiple comparisons and a p < 0.05 was considered statistically significant.

Received: 12 October 2021; Accepted: 30 May 2022
Published online: 15 June 2022

References
1. Jensen, B., Knudsen, I. M. B., Madsen, M. & Jensen, D. F. Biopriming of infected carrot seed with an antagonist, Clonostachys rosea, selected for control of seedborne Alternaria spp. Phytopathology 94, 551–560 (2004).
2. Kamou, N. N. et al. Investigating the compatibility of the biocontrol agent Clonostachys rosea IK726 with prodigiosin-producing Serratia rubidaea S55 and phenazine-producing Pseudomonas chlororaphis TozA7. Arch. Microbiol. 198, 369–377 (2016).
3. Zhang, L. et al. Investigation on the infection mechanism of the fungus Clonostachys rosea against nematodes using the green fluorescent protein. Appl. Microbiol. Biotechnol. 78, 983–990 (2008).
4. Zou, C. G. et al. Expression of a serine protease gene prc is up-regulated by oxidative stress in the fungus Clonostachys rosea: Implications for fungal survival. PLoS One 5, e13386 (2010).
5. Rodriguez, M. A., Cabrera, G., Gozzo, F. C., Eberlin, M. N. & Godeas, A. Clonostachys rosea BAFC3874 as a Sclerotinia sclerotiorum antagonist: Mechanisms involved and potential as a biocontrol agent. J. Appl. Microbiol. 110, 1177–1186 (2011).
6. Iqbal, M. et al. Natural variation of root lesion nematode antagonism in the biocontrol fungus Clonostachys rosea and identification of biocontrol factors through genome-wide association mapping. Evol. Appl. 13, 2264–2283 (2020).
7. Sun, Z. B. et al. The transcription factor-encoding gene crtf is involved in Clonostachys chloroleuca mycoparasitism on Sclerotinia sclerotiorum. Microbiol. Res. 210, 6–11 (2018).
8. Lu, B. et al. Cell wall biogenesis protein phosphatase CrSsd1 is required for conidiation, cell wall integrity, and mycoparasitism in Clonostachys rosea. Front. Microbiol. 11, 1640 (2020).
9. Sun, Z. B., Sun, M. H. & Li, S. D. Identification of mycoparasitism-related genes in Clonostachys rosea 67-1 active against Sclerotinia sclerotiorum. Sci. Rep. 5, 18169 (2015).
10. Sun, Z. B., Wang, Q., Sun, M. H. & Li, S. D. The mitogen-activated protein kinase gene Crmapk is involved in Clonostachys chloroleuca mycoparasitism. Mol. Plant Microbe Interact. 33, 902–910 (2020).
11. Eisman, B. et al. The Cek1 and Hog1 mitogen-activated protein kinases play complementary roles in cell wall biogenesis and chlamydospore formation in the fungal pathogen Candida albicans. Eukaryot. Cell 5, 347–358 (2006).
12. Yang, Q. Q., Yan, L. Y., Gu, Q. & Ma, Z. H. The mitogen-activated protein kinase kinase kinase BcOs4 is required for vegetative differentiation and pathogenicity in Botrytis cinerea. Appl. Microbiol. Biotechnol. 96, 481–492 (2012).
13. Wei, W. et al. Colletotrichum higginsianum mitogen-activated protein kinase ChMK1: Role in growth, cell wall integrity, colony melanization, and pathogenicity. Front. Microbiol. 7, 1212 (2016).
14. Gruber, S. & Zeilinger, S. The transcription factor SteI2 mediates the regulatory role of the Tmk1 MAP kinase in mycoparasitism and vegetative hyphal fusion in the filamentous fungus Trichoderma atroviride. PLoS One 9, e111636 (2014).
15. Yu, L., Xiong, D. G., Han, Z., Liang, Y. M. & Tian, C. M. The mitogen-activated protein kinase gene CcPank1 is required for fungal growth, cell wall integrity and pathogenicity in Cytospora chrysosperma. Fungal Genet. Biol. 128, 1–13 (2019).
16. Yun, Y. Z. et al. The MAPKK FgMkk1 of Fusarium graminearum regulates vegetative differentiation, multiple stress response, and virulence via the cell wall integrity and high-osmolarity glycerol signaling pathways. Environ. Microbiol. 16, 2023–2037 (2014).
17. Jagodziki, P., Tadej-Zielinska, M., Ciesla, A., Marcza?, M. & Ludwikow, A. Mitogen-activated protein kinase cascades in plant hormone signaling. Front. Plant Sci. 9, 1387 (2018).
18. Gonzalez-Rubio, G., Fernandez-Acero, T., Martin, H. & Molina, M. Mitogen-activated protein kinase phosphatases (MKPs) in fungal signaling: Conservation, function, and regulation. Int. J. Mol. Sci. 20, 1709 (2019).
19. Jiang, C., Zhang, X., Liu, H. & Xu, J. R. Mitogen-activated protein kinase signaling in plant pathogenic fungi. PLoS Pathog. 14, e1006875 (2018).
20. Zeng, F. Y. et al. A fungal cell wall integrity-associated MAP kinase cascade in Coniothyrium minitans is required for conidiation and mycoparasitism. Fungal Genet. Biol. 49, 347–357 (2012).
21. Mendoza-Mendoza, A. et al. Enhanced biocontrol activity of Trichoderma through inactivation of a mitogen-activated protein kinase. Proc. Natl. Acad. Sci. U.S.A. 100, 15965–15970 (2003).
22. Park, G., Xue, C. Y., Zheng, L., Lam, S. & Xu, J. R. Mit72 regulates infectious growth but not appressorium formation in the rice blast fungus Magnaporthe grisea. Mol. Plant-Microbe Interact. 15, 183–192 (2002).
23. Marín, M. J. et al. Different modulation of the outputs of yeast MAPK-mediated pathways by distinct stimuli and isoforms of the dual-specificity phosphatase Msg5. Mol. Genet. Genomics 281, 345–359 (2009).
24. Hamel, L. P., Nicole, M. C., Duplessis, S. & Ellis, B. E. Mitogen-activated protein kinase signaling in plant-interacting fungi: Distinct messages from conserved messengers. Plant Cell 24, 1327–1351 (2012).
25. Fields, S. & Sternglanz, R. The two-hybrid system: An assay for protein–protein interactions. Trends Genet. 10, 286–292 (1994).
26. Alcantara, A. et al. Systematic Y2H screening reveals extensive effector-complex formation. Front. Plant Sci. 10, 1437 (2019).
27. Woloschuk, R. M., Reed, P. M. M., McDonald, S., Uppalapati, M. & Woolley, G. A. Yeast two-hybrid screening of photoswitchable protein–protein interaction libraries. J. Mol. Biol. 432, 3113–3126 (2020).
28. Zhao, S. Y. et al. Screening and identification of host proteins interacting with Theileria annulata cysteine proteinase (TaCP) by yeast-two-hybrid system. Parasit. Vectors 10, 536 (2017).
29. Sun, Z. B., Sun, M. H. & Li, S. D. Draft genome sequence of mycoparasite Clonostachys rosea strain 67-1. Genome Announc. 3, e0054-15 (2015).
30. Raman, M., Chen, W. & Cobb, M. H. Differential regulation and properties of MAPKs. Oncogene 26, 3100–3112 (2007).
31. Rajagopala, S. V. Mapping the protein–protein interactome networks using yeast two-hybrid screens. J. Mol. Biol. 453, 16–13 (2015).
32. Zhang, X., Wang, Z. Y., Jiang, C. & Xu, J. R. Regulation of biotic interactions and responses to abiotic stresses by MAP kinase pathways in plant pathogenic fungi. Stress Biol. 1, 5 (2021).
33. Tanaka, S. An efficient method for the isolation of interaction-null/impared mutants using the yeast two-hybrid technique. Genes Cells 24, 781–788 (2019).
34. Kong, L. et al. Screening and analysis of proteins interacting with OsMADS16 in rice (Oryza sativa L.). PLoS One 14, e0221473 (2019).
35. He, Y. et al. Yeast two-hybrid screening for proteins that interact with PFT in wheat. Sci. Rep. 9, 15521 (2019).
36. Cao, S. H. & Yan, L. L. Construction of a high-quality yeast two-hybrid (Y2H) library and its application in identification of interacting proteins with key vernalization regulator TaVRN-A1 in wheat. BMC Res. Notes 6, 81 (2013).
37. Huang, C. X. et al. Inhibition of developmental activities of AP-1 and c-Jun by a new zinc finger protein ZNF394. Biochem. Biophys. Res. Commun. 320, 1298–1305 (2004).
38. Liu, H. et al. ZNF411, a novel KRAB-containing zinc-finger protein, suppresses MAP kinase signaling pathway. Biochem. Biophys. Res. Commun. 320, 45–53 (2004).
39. Li, X. Q. et al. ZNF32, a novel human C2H2 Kruppel-like zinc-finger protein, regulates transcriptional activation in MAPK signaling pathways. Biochem. Biophys. Res. Commun. 325, 1383–1392 (2004).
40. Zhang, H. et al. The C2H2-type zinc finger protein ZFP182 is involved in abscisic acid-induced antioxidant defense in rice. J. Integr. Plant Biol. 54, 500–510 (2012).
41. Kob, R. et al. Regulation of the anaphase-promoting complex by the COP9 signalosome. Cell Cycle 8, 2041–2049 (2009).
42. Tong, X. W., Chen, B., Huang, L. H., Feng, Q. L. & Kang, L. Proteomic analysis reveals that COP9 signalosome complex subunit 7A (CSN7A) is essential for the phase transition of migratory locust. Sci. Rep. 5, 12542 (2015).
43. Brockway, H., Balukoff, N., Dean, M., Alleva, B. & Smolikova, S. The CSN/COP9 signalosome regulates synaptosomal complex assembly during meiotic prophase I of Caenorhabditis elegans. PLoS Genet. 10, e1004757 (2014).
44. Liang, L. et al. Cloning and homology modeling of a serine protease gene from the nematode Heterodera schachtii. Plant Biol. 32, 883–909 (2010).
45. Pozo, M. J. et al. Functional analysis of ipr1, a serine protease–encoding gene in the biocontrol agent Trichoderma virens. Fungal Genet. Biol. 41, 336–348 (2004).
46. Iqbal, M. et al. Comparative evolutionary histories of fungal proteases reveal gene gains in the mycoparasitic and nematode-parasitic fungus Clonostachys rosea. BMC Evol. Biol. 18, 171 (2018).
47. Chavan, S. B. & Deshpande, M. V. Chitinolytic enzymes: An appraisal as a product of commercial potential. Biotechnol. Prog. 29, 404–413 (2013).
48. Zhang, A. E. et al. Novel bacterial β-N-acetyl glucosaminidase from Chitinimonas sp. possessing transglycosylation and reverse hydrolysis activities. Biotechnol. Biofuels 13, 115 (2020).
49. Lopes-Mondéjar, R. et al. Evaluation of the effect of chitin-rich residues on the chitinolytic activity of Trichoderma harzianum: In vitro and greenhouse nursery experiments. Pestic. Biochem. Physiol. 103, 1–8 (2012).
50. Huang, T. K. et al. Identification of downstream components of ubiquitin-conjugating enzyme PHOSPHATE2 by quantitative membrane proteomics in Arabidopsis roots. Plant Cell 25, 4044–4060 (2013).
51. Zhang, Y. H., Gao, H. L., Ma, G. Z. & Li, S. D. Mycoparasitism of Gliocladium roseum 67-1 on Sclerotinia sclerotiorum. Acta. Phytopathol. Sin. 34, 211–214 (2004).
52. 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 work was supported by the National Natural Science Foundation of China (32072478), the National Key Research and Development Program of China (2019YFD1002000), and the China Agriculture Research System of MOF and MARA (CARS-23-D05).

Author contributions
M.S., S.L. and B.L. conceived and designed research; B.L. performed the experiments and wrote the manuscript; B.L. and L.F. analysed data; M.S. and S.L. provided fundings and reviewed the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-13899-3.

Correspondence and requests for materials should be addressed to M.S.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
