The Polyketide Synthase-Encoding Gene Crpks is Involved in Clonostachys Rosea Chlamydospore Formation

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Research

Abstract

Clonostachys rosea is an excellent agent for biocontrol of numerous plant fungal diseases. Polyketide synthases (PKSs) are widely distributed in plants and microorganisms and synthesize various types of polyketides. In this study, a type I PKS-encoding gene, crpks, was cloned and identified from the C. rosea 67-1 genome, and the biological function was investigated through gene knockout. The results showed that crpks deletion did not affect C. rosea morphology, ability for parasitism of sclerotia and the capacity for biocontrol of soybean Sclerotinia white mold, but had a marked influence on the chlamydospore formation ability of C. rosea. After cultivation for 48 and 72 h, chlamydospore production by Δcrpks was increased by 70.1% and 47.6%, respectively, compared to that of the wild-type strain. These data indicate that crpks is involved in C. rosea chlamydospore formation and provide useful insights into the molecular mechanisms of chlamydospore formation in C. rosea.

Introduction

Polyketide synthases (PKSs), which are widely distributed in plants and microorganisms, synthesize various types of polyketides, including antibiotics, toxins, and native pigments. PKSs can be classified into three types according to structure and catalytic mechanism. Type I PKSs are huge proteins containing multiple functional domains linked by covalent bonds (Smith and Tsai 2007). Type II PKSs represent a complex enzymatic system that includes several independent functional proteins (Hertweck et al. 2007). Type III PKSs comprise only ketone condensation enzymes (Austin and Noel 2003). Generally, fungal PKSs domains, such as acyltransferase (AT) and ketosynthase (KS), are huge proteins containing a major acyl carrier protein (ACP) domain, together with other catalytic activity domains; therefore, fungal PKSs are regarded as type I PKS (Kroken et al. 2003).

PKSs are involved in a variety of biological functions in fungi, the most common of which is the synthesis of secondary metabolites during fungal growth. Secondary metabolite production has been shown to be lost in the absence of PKS-encoding genes. For example, the ability to produce the polyketides melanin and 4-methyl-5-pentylbenzene-1,3-diol was lost in PKS-deficient *Pestalotiopsis microspore* mutants (Narita et al. 2011; Yu et al. 2015). Deletion of the PKS-encoding gene influenced the production of conidial pigment in *Trichoderma harzianum* 88, with the color of the conidia in mutants changing from green to pink (Yao et al. 2016). In addition to the synthesis of secondary metabolites, PKSs participate in fungal spore growth and development. Disruption of PKS-encoding genes resulted in loss of the ability to form spores in *Chaetomium globosum* (Hu et al. 2012), incomplete multicellular conidia formation in *Pestalotiopsis fici* (Zhang et al. 2017), and delayed fruiting body maturation in both *Sordaria macrospora* and *Neurospora crassa* (Nowrousian 2009). To some extent, PKSs also regulate fungal virulence. Disruption of PKS-encoding genes significantly reduced the virulence of *Beauveria bassiana* against beet armyworms (Toopaang et al. 2017), *Penicillium marneffei* against mice (Woo et al. 2010), and decreased the virulence of *T. reesei* against *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Alternaria alternate* (Atanasova et al. 2013).

The mycoparasite *Clonostachys rosea* mediates excellent biocontrol of numerous plant diseases such as gray mold and whitefly, and shows great potential in promoting crop growth and controlling plant diseases (Cota et al. 2008; Schöneberg et al. 2015; Keyser et al. 2016; Ruiz-Jiménez et al. 2017). *C. rosea* could produce chlamydospores during its life cycle. Chlamydospores are of great significance in biocontrol and commercialization of biopesticides as well as fungal survival. As a mycoparasite, *C. rosea* could intertwine the mycelium of host pathogens, and secreted cell wall degrading enzymes like chitinase and glucanases or toxins to suppress or even kill host pathogens (Karlsson et al. 2017). A number of PKSs have been identified in *C. rosea* IK726 through bioinformatics analysis, and are predicted to be involved in biosynthesis of the pink, yellow, orange, and brown pigments, then compounds polyketide Clonorosein A-D were isolated (Karlsson et al. 2015; Fatema et al. 2018). In our previously study, we sequenced the transcriptome of *C. rosea* 67-1 mycoparasitizing sclerotia of *S. sclerotiorum* and found a PKS-encoding gene *crpks* was differentially expressed under *C. rosea* mycoparasitism (Sun et al. 2015a). In this study, we cloned and identified *crpks* from the *C. rosea* 67-1 genome (Sun et al. 2015b). Using gene knockout technology, we also characterized the biological functions of *crpks* expression on colony morphology, sporulation ability, mycoparasitism and biocontrol capacity against soybean white mold.

Methods

Strains and *crpks* gene

C rosea 67-1 was originally isolated from a vegetable plantation in Hainan Province, China. *S. sclerotiorum* was isolated from soybean affected by Sclerotinia white mold in Heilongjiang Province (Zhang et al. 2004). All strains are preserved in the Biocontrol of Soilborne Diseases Laboratory of the Institute of Plant Protection, Chinese Academy of Agricultural Sciences.

The polyketide synthases encoding gene *crpks* was isolated from the genome of *C. rosea* 67-1 and its sequence was deposited in GenBank under accession number KY701733.

Gene cloning and bioinformatics analysis

Full-length *crpks* was cloned from the genome of *C. rosea* 67-1 using the *PSnei*F and *PSnei*R primer pair (Table 1) with 25 µL reaction system contained 2 µL DNA template, 1 µL *PSnei*F and *PSnei*R primer pairs, 12.5 µL PCR mix and 8.5 µL ddH$_2$O. The PCR program was conducted as follows: 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min, with a final extension at 72°C for 10 min.

The molecular weight and isoelectric point of the Crpks protein were calculated using the ExPASy program. The hydrophobicity profile, signal peptide and transmembrane regions prediction were investigated using the ProtScale program, Signal 4.1 procedure and TMPRED software, respectively (Hofmann and Stoffel 1993; Gasteiger et al. 2005; Petersen et al. 2011). The functional domains of Crpks were detected using antiSMASH fungal version (Blin et al. 2019). Sequences that were closely similar with Crpks were identified by NCBI Blast searches and used for construct phylogenetic tree. Phylogenetic

tree of Crpks was conducted by using MEGA version 5.1 with the neighbor-joining method under Kimura two-parameter model and bootstrap values were based on 1000 replications (Saitou and Nei 1987).

Gene knockout and validation

The pKH-KO plasmid, containing a *trpc* promoter, a hygromycin B resistance gene, and two uracil-specific excision reagent cloning sites, was used for construction of the *crpks* knockout vector as previously described (Sun et al. 2018), with the *PUF/PUR* and *PDF/PDR* primer pairs used for amplification of the upstream and downstream regions (Table 1). Knockout vector was verified using electrophoresis and DNA sequencing.

Transformation of vectors into *C. rosea* was conducted as previous described with minor revised (Sun et al. 2017). Fresh *C. rosea* spores were incubated on potato dextrose (PD) broth and cultivated at 26 °C on a rotary shaker with the speed of 180 rpm for 12 h. Then mycelium of *C. rosea* were collected by a 125-μm sterile sieve, and then washed several times with sterile distilled water and 0.7 mol/L NaCl. The cell wall of *C. rosea* mycelium was hydrolyzed with 40 mg/mL snail enzymes for 3 h on a rotary shaker with the speed of 100 rpm at 28 °C. The protoplasts were filtered with a sterile microfiber filter and then collected by centrifugation at 1500×g for 10 min. Finally the protoplasts were kept in STC buffer (50 mL 1 mol/L Tris-HCl with pH value of 8.0, 200 g sucrose, 5.55 g of $CaCl_2$ in 1 L distilled water) with the concentration of 10^7 protoplasts/mL.

Knockout vector was gently added into protoplasts suspension of *C. rosea* and placed on ice for 20 min. Then added PTC solution (10 mL 1 mol/L Tris-HCl with pH value of 8.0, 20 mL 2.5 mol/ L $CaCl_2$, and 400 g polyethylene glycol 4000 in 1L distilled water) and keep room temperature for 20 min. The mixture were then incubated into TB3 (200 g sucrose, 3 g yeast extract and 3 g casein acid hydro-lysate in 1 L distilled water) and cultured on a rotary shaker with the speed of 100 rpm at 26 °C for 16 h. Then the mixture was centrifuged at 2500×g for 10 min and the precipitation was suspended in STC buffer. Finally the suspension was mixed into TB3 plate with 300 μg mL^{-1} of hygromycin B and cultivated at 26 °C for 3-7 days. Mutants obtained from TB3 plates were transferred into potato dextrose agar (PDA) plates containing 300 μg/ml hygromycin B for screening, and then cultured for three generations to verify genetic stability.

Genomic DNA of mutants was extracted using a Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Co., Ltd, Hangzhou, China). PCR amplification was performed to validate positive mutants using the *PSnei*F and *PSnei*R primer pair for which it was not possible to amplify the *crpks* open reading frame (ORF), F*PS*F and F*PS*R primer pair for which it can amplify the fragments beyond upstream and downstream regions (-3893 bp) were considered to positive deletion mutants designated as Δ*crpks*.

Morphology assay

Fresh spores of the wild-type strain (WT) and the positive mutant with the equal concentration were inculcated and cultured on PDA plates at 26 °C for 1 week. The morphology of colonies and mycelia were

observed, and the diameter of colonies was measured using Vernier calipers. Fresh spores of the WT and the positive mutant with the equal concentration were inoculated into PD broth and cultivated at 26 °C for 36 h on a rotary shaker with the speed of 180 rmp. Then 2% fermentation broth was transferred into chlamydospores production medium as previously described (Sun et al. 2018). Chlamydospores were collected after 48 and 72 h and counted under a microscope. The experiment was conducted with three replicates.

Mycoparasitism *S. sclerotiorum* sclerotia

S. sclerotiorum sclerotia were collected as previously described (Sun et al. 2015c). Sclerotia were surface-sterilized with 1% NaClO for 3 min, and immersed in *C. rosea* spore suspensions (10^7 spores/ml) for 10 min. Then sclerotia were placed on sterile filter paper for moist cultured in a Petri dish at 26°C for 7 days. The severity of Sclerotia parasitism was investigated on a 4-grade scale: 0 = no *C. rosea* was detected on the surface of sclerotia; 1 = rare hyphae of *C. rosea* was observed on sclerotia; 2 = sclerotia were covered with hyphae of *C. rosea* but keep firm; 3 = sclerotia were covered with hyphae of *C. rosea* and exhibit softening. The experiment was conducted with three replicates.

Biocontrol efficacy against soybean Sclerotinia white mold

Field trials of biocontrol efficacy were performed in soybean plants at the 9-compound leaves stage of development. Leaves were sprayed with *C. rosea* spore suspensions (5×10^6 spores/ml) and then with an equal volume of *S. sclerotiorum* mycelial suspension after 2 h. Carbendazim and sterile distilled water were used as positive and negative controls, respectively. The disease index of soybean Sclerotinia white mold was investigated after 7 days using a previously defined 9-grade scoring system: 0 = no symptoms; 1 = less than 5%; 3 = 5−10%; 5 = 11−25%; 7 = 26−50%; 9 = over 50%. The experiment was conducted with three replicates.

Statistical analysis

The statistical software SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) was used for analysis of variance. Duncan's multiple range test was used to compare the means of biocontrol efficacy of *C. rosea* against soybean Sclerotinia white mold. *t*-tests were used to compare the means of other treatment groups. $P < 0.05$ was considered to indicate statistical significance.

Results

Characterization of Crpks

The full-length *crpks* sequence contained a 7,584-bp ORF encoding a 277-kDa protein consisting of 2,527 amino acids. Crpks was identified as a hydrophilic protein with an isoelectric point of 5.77, and no transmembrane region or signal peptide was detected (Fig. 1A, 1B). Functional domains searches showed that Crpks had a PKS domain, and was classified as a type I member of the PKS family (Fig. 1C).

The domain organization of Crpks contained AT, DH, cMT, ER and KR. Fourteen sequences with high similarity of Crpks were selected to construct phylogenetic tree, phylogenetic analysis demonstrated that Crpks is a member of the polyketide synthetase family analysis (Fig. 2).

Validation of *crpks*-deletion mutants

A total of 97 mutants that grew stably on hygromycin selection plates were obtained; however, only one mutant was verified as a positive *crpks*-deletion mutant according to PCR amplification and DNA sequencing, with colony diameters between *C. rosea* 67-1 (63.5±1.1 mm) and $\Delta crpks$ (63.9±1.6 mm). However, an obvious increase in chlamydospore production was detected in $\Delta crpks$ compared with *C. rosea* 67-1. After cultivation for 48 and 72 h, chlamydospore production by *C. rosea* 67-1 was $(2.3\pm0.1)\times10^7$ and $(6.1\pm0.3)\times10^7$ spores/ml, respectively, compared with $(3.9\pm0.2)\times10^7$ and $(8.9\pm0.2)\times10^7$ spores/ml, respectively, by $\Delta crpks$ (Table 2).

Mycoparasitism of *S. sclerotiorum* sclerotia by $\Delta crpks$

After 7 days, no significant difference in the mycoparasitic ability of 67-1 and $\Delta crpks$ was observed and sclerotia treated with the 67-1 and $\Delta crpks$ exhibited clear symptoms of Sclerotia white mold. Both strains induced the highest level of mycoparasitism (grade 3), indicating that *crpks*-deletion had no effect the mycoparasitic ability of *C. rosea* (Fig. 3).

Biocontrol efficiency of $\Delta crpks$ against soybean Sclerotinia white mold

Serious symptoms of white mold were observed on soybean leaves after incubation with *S. sclerotiorum* for 7 days. $\Delta crpks$ exhibited an excellent biocontrol efficacy against soybean Sclerotinia white mold (72.8±6.1%), with similar effects mediated by 67-1 (74.4±4.9%) and carbendazim (71.7±5.4%). These data suggested that *crpks* deletion did not influence the biocontrol efficacy of *C. rosea* (Table 2).

Discussion

PKSs play an essential role in secondary metabolite synthesis. Recently, the involvement of PKSs in other biological functions such as growth, development, and virulence have been reported in fungi. In our previous study, we sequenced the whole genome of *C. rosea* 67-1 (Sun et al. 2015b). In the present study, we cloned a type I PKS-encoding gene, *crpks*, from the *C. rosea* genome and investigated the biological functions of *crpks* using gene knockout technology. Our results showed that *crpks*-deletion had no effect on the biological functions of *C. rosea*, with the exception of chlamydospore production, which was significantly increased. Thus, our findings indicate that *crpks* plays an essential role in chlamydospore formation. To the best of our knowledge, this is the first report of polyketide synthetase encoding gene has close relationship with fungal chlamysospore production.

PKSs play different role in the virulence of various types of fungi. In *Magnaporthe oryzae*, expression of the PKS-encoding gene *ALB1* was positively correlated with virulence, and the pathogenic ability of *M.*

oryzae in rice and barley was lost through targeted gene disruption (Oh et al. 2008). A similar phenomenon has also been reported in *Aspergillus fumigatus* and *A. alternate* (Tsai et al., 1998; Miyamoto et al. 2010). However, in *Ustilago maydis* and *Metarhizium robertsii*, host pathogenicity had no relationship with PKS gene expression and the virulence was not impaired in PKS-encoding gene deficient mutants compared with that of the WT strain (Chen et al. 2015; Islamovic et al. 2015). Totally 29 polyketide synthetase encoding gene exist in the genome of *C. rosea* 67-1. Among them, *crpks* was differentially expressed under *C. rosea* mycoparatizing sclerotia. However, *crpks* disruption did not influence the virulence against host pathogens, including mycoparasitism of sclerotia, and the capacity for biocontrol of soybean Sclerotinia white mold. That might due to the existence of PKS gene cluster. Although *crpks* was disrupted, other genes in PKS gene cluster might remedy and keep normal biocontrol ability for *C. rosea*. Future work should detect the expression level of other genes within the same PKS gene cluster after *crpks* deletion. Then knockout all the genes existed in PKS gene cluster and investigate the relationship between PKS genes and *C. rosea* biocontrol ability.

Chlamydospores are a specific structure with a thick cell wall and high resistance to harsh environments (Armengol et al. 1999; Kang et al. 2016). Chlamydospores have the ability to form and survive under unfavorable conditions, including unsuitable pH values and temperatures (Eisman et al. 2006). In this study, the yield of chlamydospores was closely related to the expression of *crpks*. Disruption of *crpks* significantly increased the quantity of *C. rosea* chlamydospores. Li et al. found low concentrations of metabolic compounds could induce the chlamydospores in *C. rosea* (Li et al. 2005). We deduced that deletion of *crpks* might reduce the quantity of crucial metabolites that related to chlamydospores production, therefore increase the yield of chlamydospores. In the future, we should analysis the difference of metabolites between the mutant and wild strain, and seek out the expected metabolites that associated with chlamydospores production. Besides, future work also should investigate the differentially expressed genes between the mutant and wild strain through transcriptome sequencing and analysis, and understand the molecular mechanism of *crpks* on chlamydospore formation of *C. rosea*.

Conslusion

we investigated the biological function of *crpks* in *C. rosea* 67-1, and found that *crpks*-deletion had no effect on *C. rosea* morphology, sclerotia parasitic ability and capacity for biocontrol of soybean Sclerotinia white mold, while chlamydospore production was significantly increased. This is the first report of the close relationship between PKS-encoding gene expression and fungal chlamydospore production. This information provides further insights into the molecular mechanism of chlamydospore formation.

Declarations

Author's contributions

MHS, SDL and ZBS. design this research. ZBS, QW and YWZ performed this experiment. ZBS and QW carried out data analysis. ZBS wrote original manuscript. MHS and SDL contributed to manuscript revision. All authors have participated in this research and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Availability of data and materials

All data used in this study are include in this article.

Consent for publication

Not applicable

Ethics approval and consent to participate

Not applicable

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Tables

Table 1: Primers used in this study.

| Primer	Sequence (5'-3')	Purpose
*PSnei*F	ATGGCCCCTTTCATGGAGGCGTCAG	Amplification of *crpks*
*PSnei*R	TCAAGAGGCATTCTTTGCTTGCATCTT	
*PU*F	GGTCTTAAUCATGCTCTCATCCAACTGTTCGA	Amplification of upstream regions
*PU*R	GGCATTAAUCATATAAATGTACGTAGCGTAAC	
*PD*F	GGACTTAAUCCTTAAATTGTTTTTTATCTCTT	Amplification of downstream regions
*PD*R	GGGTTTAAUGATTGGGTTGAACCTGAGACCAC	
F*PS*F	GACAACGGCGCTCGAGCTTACAAAGGTCTTG	Verification of positive mutants
F*PS*R	TGTCGACTGGGAAGCTGAGATATCCGATCA	

Table 2: Analysis of biological characters of C. rosea 67-1 and Δcrpks.

Biological characteristic	WT	*Δcrpks*
48-h Chlamydospore production (1× 10^7 spores/ml)	2.3±0.1[a]	3.9±0.2[b]
72-h Chlamydospore production (1× 10^7 spores/ml)	6.1±0.3[a]	8.9±0.2[b]
Mycoparasitism ability (grade)	3[a]	3[a]
Biocontrol efficacy of *C. rosea* against soybean Sclerotinia white mold (%)	74.4±4.9[a]	72.8±6.1[a]

Identical letters in the same line indicate no significant differences ($P < 0.05$). WT, wild-type strain; Mutant, deletion mutant. Data represent the means of three replicates.
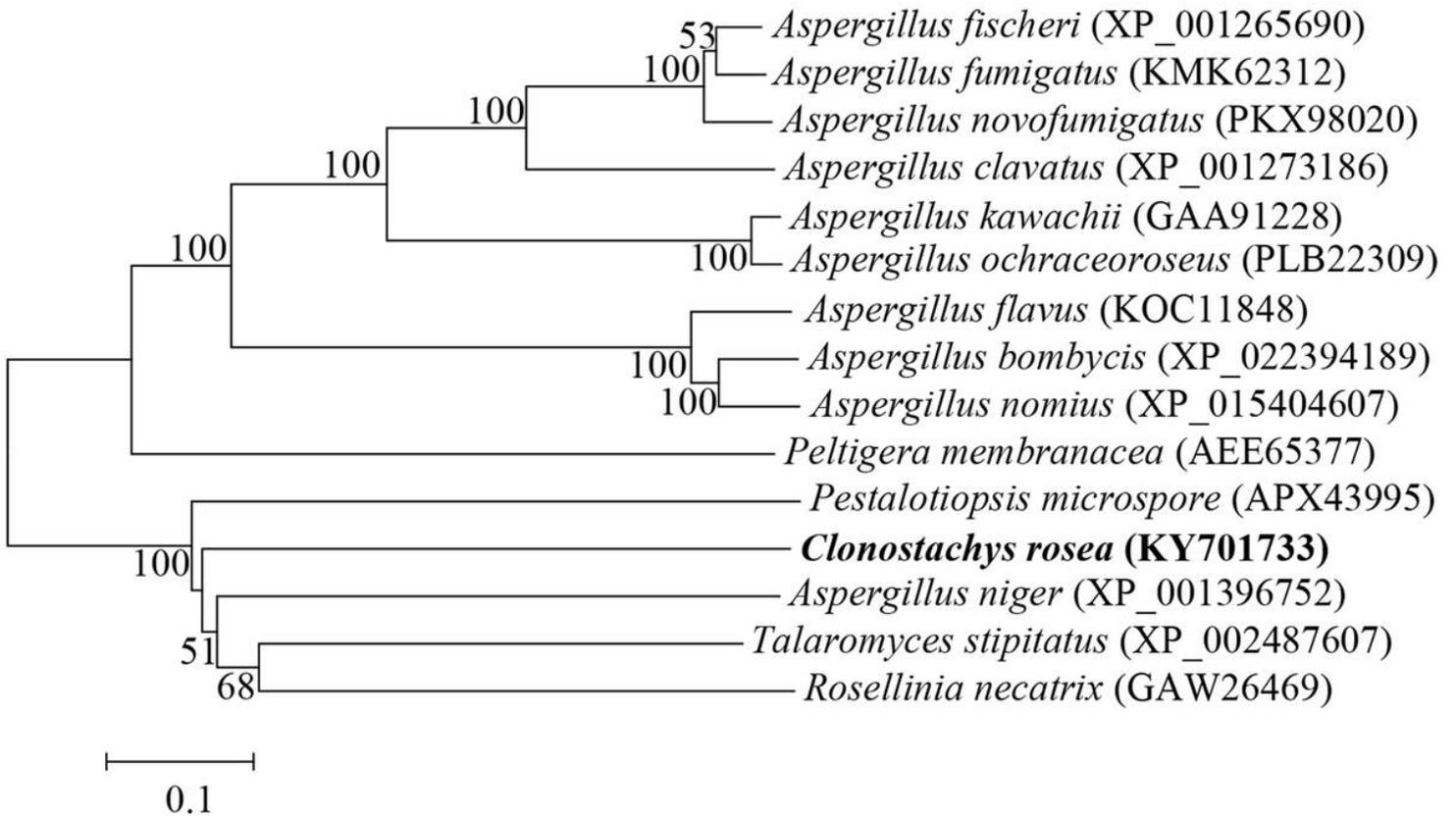
Figures

A
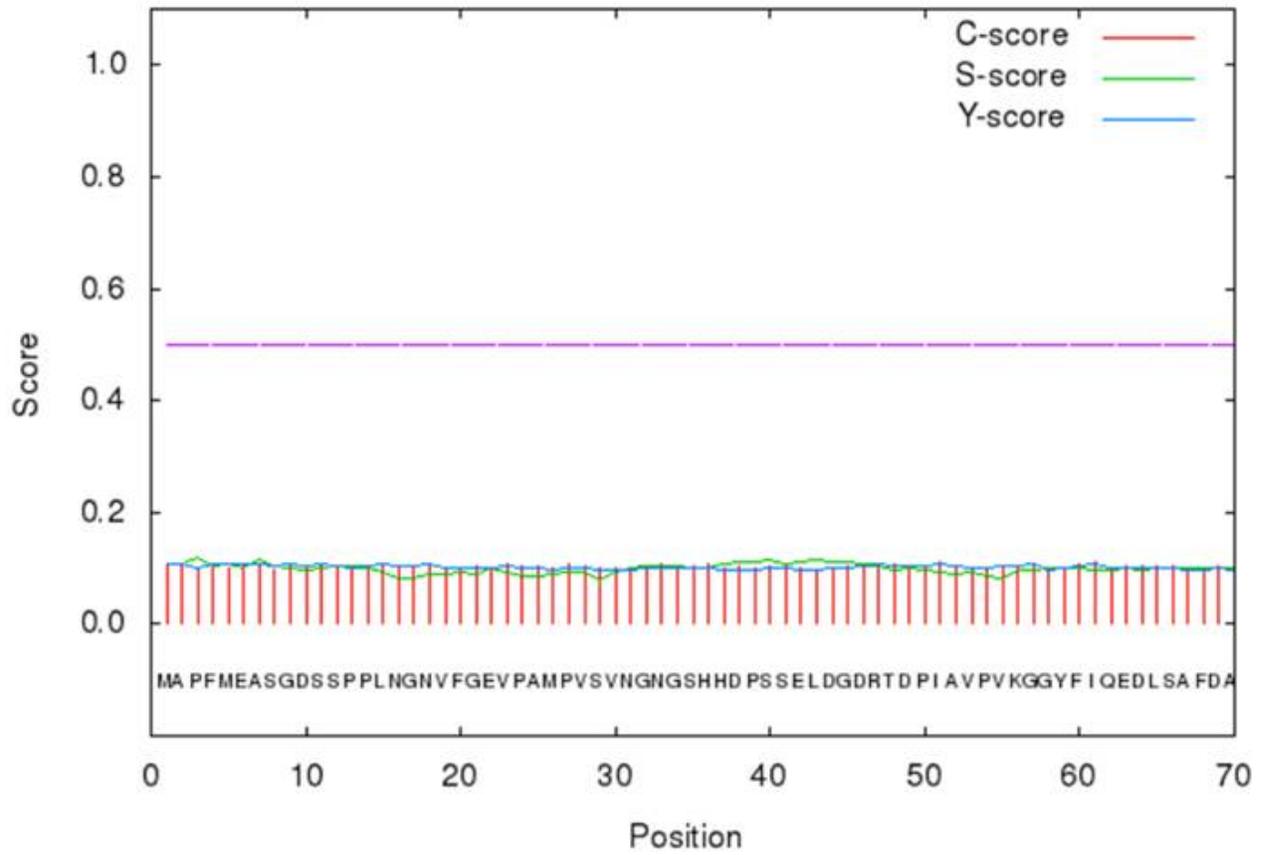
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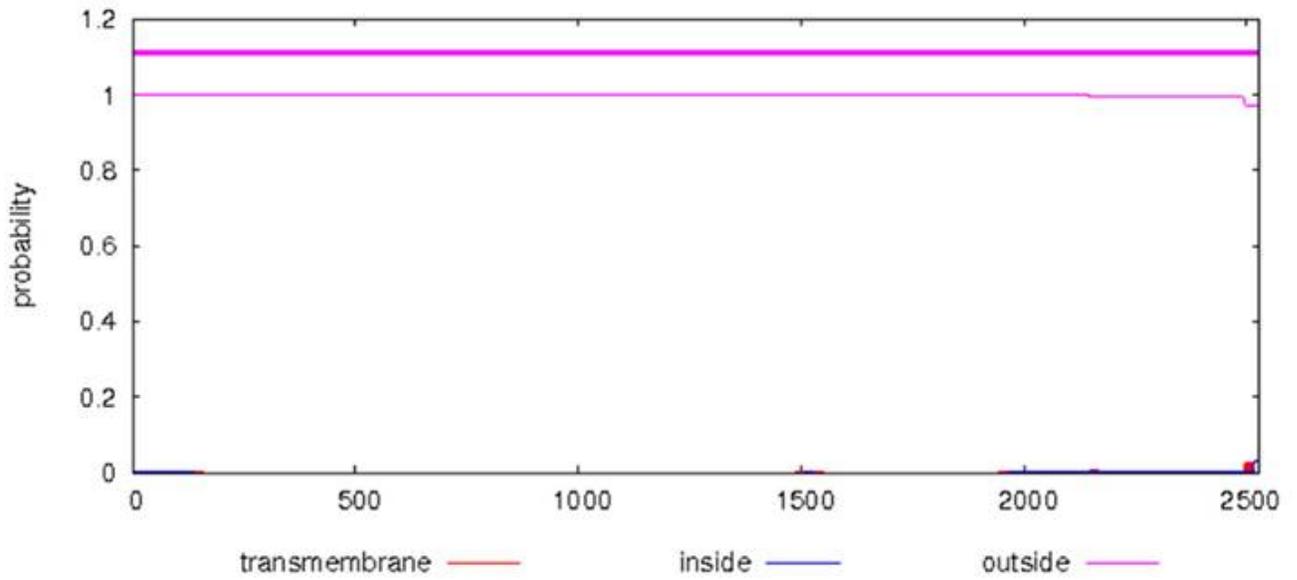
C
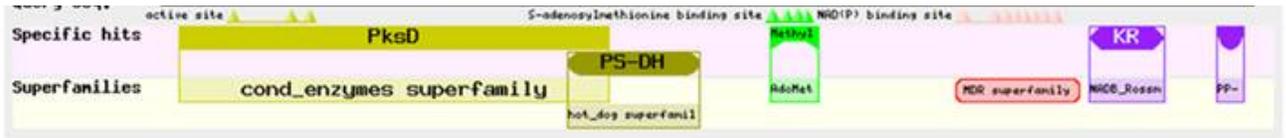
Figure 1

Bioinformatic analysis of crpks. A. Signal peptide analysis. B. Transmembrane region analysis. C. Functional domains analysis.

Figure 2

Neighbor-joining phylogenetic tree of crpks from C. rosea. Fourteen sequences with high similarity of Crpks were selected to construct phylogenetic tree. Numbers in parentheses represent the sequence accession numbers in GenBank. Bar (0.1) represents sequence divergence.

Figure 3

Mycoparasitism of C. rosea on sclerotia of S. sclerotiorum. WT: wild type strain; Δcrpks: crpks-deletion strain.