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Two cerato-platanin proteins FocCP1 interact with MaPR1 and contribute to virulence of *Fusarium oxysporum* f. sp. *cubense* to banana

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ABSTRACT

Fusarium oxysporum f. sp. *cubense* tropical race 4 (Foc TR4) causes destructive wilt disease to banana cultivation. However, knowledge of mechanisms in virulence regulation of Foc TR4 is still inadequate. In our previous work, two hypothetical FocCP1 proteins that belong to the cerato-platanin (CP) family were identified in the secretome of Foc TR4. In the present study, knock-out mutants of two FocCP1 were constructed and functions of two proteins were investigated. The results showed that the two FocCP1 play important roles in regulation of full virulence but are not required for vegetative growth or conidiation of the pathogen; besides, there is a functional redundancy between the two FocCP1. Moreover, we found that FocCP1 proteins interact with MaPR1 proteins through yeast two-hybrid and BiFC test. Our results suggest that FocCP1 proteins are important for the virulence of Foc TR4 and might inhibit the antifungal activity of MaPR1 by directly interacting with them.

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KEYWORDS
Fusarium oxysporum f. sp. *cubense*; cerato-platanin; virulence; interaction; PR1

Introduction

Fusarium oxysporum f. sp. *cubense* (Foc) is the causal agent of banana (*Musa* spp.) wilt disease (also named as 'panama disease'). Four races of Foc have been recognized so far, among which Foc tropical race 4 (Foc TR4) could infect Cavendish banana, the main commercial banana cultivar worldwide, and lead to serious economic loss (Ploetz 2005). Because of the strong stress resistance of the Foc TR4, there is still no effective strategies for management of the soil borne pathogen (Ordonez et al. 2015). Thus, figuring out the mechanism in regulating the virulence of Foc TR4 might provide some clues for controlling the banana wilt disease.

During the long periods of co-evolution with plant hosts, fungal pathogens have evolved complex strategies to successively infect and colonize plant host, including secreting cell-wall-degrading enzymes (Kikot et al. 2009), production of toxins (Desjardins and Proctor 2007), and secreting small proteins called effectors to overcome plant immune systems (Chisholm et al. 2006). In *F. oxysporum* f. sp. *lycopersici* (Fol), a group of effectors named as SIX (secreted in xylem) were firstly identified in the xylem sap proteome of tomato plantlets (Houterman et al. 2007; Rep et al. 2002). Further investigation revealed that SIXs play important roles in regulating virulence and pathogen–host interaction of Fol (Houterman et al. 2009; Rep et al. 2004; van der Does et al. 2008). However, there is still limited knowledge about effectors in Foc TR4. In our previous work, the transcriptional regulator SGE1 (SIX Gene Expression 1) and the effector SIX8 were found to be required for virulence of Foc TR4 to banana plant (An et al. 2019; Hou et al. 2018). In addition, a series of effector candidates were identified in the secretome of Foc TR4 (Zhao et al. 2020); among these proteins, two candidates, FOIG_10415 and FOIG_07409, were identified as cerato-platanin family proteins (CP).

The CP protein was first found in culture filtrates of *Ceratocystis fimbriata* (Pazzagli et al. 1999), and was subsequently found only produced by pathogenic and nonpathogenic filamentous fungi (Chen et al. 2013). The CPs are highly conserved proteins composed of 105–134 amino acid residues, containing a signal peptide of 14–18 residues. The distinctive feature of CPs is the presence of four cysteines which form two disulfide bridges, conferring stability of CPs to high temperatures and acidic media (Pazzagli et al. 2014). Function analysis suggested that CPs are likely playing a dual role in fungi, exerting functions in the fungal cell wall by binding to chitin and N-acetylglucosamine (GlcNac) oligomer, or acting as elicitor or virulence factor in plant colonization. In *C. fimbriata*, CP protein is located in the cell wall of ascospores, conidia and hyphae (Boddi et al. 2004). In *Trichoderma harzianum* and *C. fimbriata*, CPs could bind to fungal chitin oligomers and help the pathogen escaping from recognition by host receptors (Gomes et al. 2016; Pazzagli et al. 2014). CP proteins MSP1, BcSPl1, and SsCP1 are required for virulence of *Magnaporthe oryzae*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum* to plant hosts; moreover, these CP proteins could also trigger hypersensitive response (HR)-like cell death and elicit defense responses of the plant (Frias et al. 2011; Jeong et al. 2007; Wang et al. 2016; Wang et al. 2018; Yang et al. 2009). However, Sm1 from *Trichoderma virens* did not induce cell death (Salas-Marina et al. 2015). Further study showed that SsCP1 directly interact with pathogenesis-related protein PR1 in *Arabidopsis thaliana*, suggesting that SsCP1 might inhibit the antifungal activity of PR1 (Yang et al. 2018). BcSpl1 from *B. cinerea* could even induce

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the systemic acquired resistance (SAR) in an salicylic acid (SA) dependent way (26). Transgenic ectopic expression of MgSM1 from *M. oryzae* confers broad-spectrum disease resistance in *Arabidopsis* (Yang et al. 2009). Recently, FOIG_10415 was identified in Foc TR4 and designated as FocCP1; and the purified FocCP1 could trigger HR and improve disease resistance in tobacco leaves (Li et al. 2019). However, whether FocCP1 proteins are required for full virulence of Foc TR4 and the possible mechanism are still unknown. In the present study, single and double gene knock-out mutants of the two FocCP1 coding genes were generated; in addition, the biological functions of the two FocCP1 proteins and their possible target proteins in the host plant were identified.

Materials and methods

Bioinformatics analysis

Reference sequences of CPs from other fungi were retrieved from the NCBI GenBank database. Protein homology modeling was performed using Clustal X2.0 (Larkin et al. 2007), and the bootstrap neighbor-joining phylogenetic tree was constructed using MEGA X (Kumar et al. 2018). Signal peptides were predicted with SignalP 5.0 (Almagro Armenteros et al. 2019).

Fungal strains and culture conditions

Wild type *F. oxysporum* f. sp. *cubense* TR4 strain II5 (NRRL#54006) was maintained on potato dextrose agar (PDA) medium or Malt extract agar medium (Oxoid, Basingstoke, England) at 28°C; and complete medium was employed for liquid culture.

Gene replacement

A Split-Marker system was used for gene knock-out the strategies were showed in Figure 2(A). Neomycin phosphotransferase gene (*NPT*II) conferring resistance to Geneticin (G418) and hygromycin phosphotransferase gene (*HPT*) were used as the selection markers. The upstream (5′) and downstream (3′) flanking fragments of two target genes were amplified from genomic DNA using primer pairs CP1a-5F/5R, CP1a-3F/3R, CP1b-5F/5R, and CP1b-3F/3R. Then the fragments were ligated into the multiple cloning sites of the vectors pBS-*NPT*II and pBS-*HPT* (using pBluescript KS as backbones) which contain *NPT*II and *HPT*, respectively. The flanking sequences with truncated resistant gene sequence were amplified with primer pairs CP1a-5F/NPTspl-R, NPTspl-F/CP1a-3R, CP1b-5F/HPTspl-R, and HPTspl-F/CP1b-3R, respectively. After that, the purified fragments for FocCP1a or FocCP1b knock-out were mixed in equal molar quantities and used for protoplast transformation. All the primers used were listed in Table S1.

Transformation of Foc TR4, PCR diagnosis, and single conidia purification

Foc TR4 wild type was used as recipient strain to construct two *FocCP1* genes (named as *FocCP1a* and *FocCP1b*) knock-out mutants. Protoplasts of Foc TR4 were prepared and transformed as described in our previous work (Hou et al. 2018), using regeneration medium containing 100 μg mL^{-1} G418 or 300 μg mL^{-1} Hygromycin B (Sigma-Aldrich, St Louis, MO, USA) to select the transformants. For the construction of the double mutant, the identified *FocCP1a* knock-out mutant was used as recipient strain, and both G418 and Hygromycin B were used to select the transformants. The transformants were further analyzed by a two-round PCR diagnosis to confirm the correct integration of the recombinant fragments into the target locus (Figure 2(A)), using primer pairs with one primer being located out the flanking fragment, and the other in the selection marker gene. For diagnosis of the FocCP1a knock-out mutants, primer pairs CP1a-SF/NPTII-SR and NPTII-SF/CP1a-SR were used; for the FocCP1b knock-out mutants, primer pairs CP1b-SF/HPT-SR and HPT-SF/CP1b-SR were used. Homokaryotic mutants were obtained by two-round single ascospore isolation. Then the homokaryotic mutants were further confirmed by PCR amplification of the FocCP1a or/and FocCP1b coding sequence.

Growth and conidiation assay

The Foc TR4 strains were inoculated on the complete medium and minimal medium, respectively; after grown for five days, the colony diameters were recorded and the growth rates were calculated. Conidia of Foc TR4 strains were inoculated into 50 mL liquid complete medium and make initial concentration of 10^3 conidia mL^{-1}. After incubation at 28°C, 150 rpm for four days, the conidia concentrations of cultures were calculated under microscope.

Inoculation of banana plantlets and Virulence assay

Banana plantlets (Musa acuminata L. AAA group, 'Brazilian') were obtained from the Tissue Culture Center of Chinese Academy of Tropical Agricultural Sciences, and were cultured in glasshouse. Foc TR4 strains were cultured in the liquid complete medium for three days, and the conidia were collected, washed and resuspended with ddH_2O to a final concentration of 10^6 conidia mL^{-1}. Then each banana root was inoculated with 200 mL of the conidia suspension. After inoculation for four weeks, the disease symptoms of banana pseudostem were recorded. The disease scores were defined as follows: 0 (no symptoms), 1 (some brown spots in the inner rhizome), 2 (less than 25% of the inner rhizome showed browning), 3 (up to 3/4 of the inner rhizome showed browning), and 4 (entire inner rhizome were dark brown). A total of 20 banana plantlets were used for each treatment. Differences in the distributions of disease scores between treatments were tested for statistical significance by Mann–Whitney tests. The experiment was conducted twice.

Yeast two-hybrid system

FocCP1a and FocCP1b without signal peptide (SP) were introduced into pGBKT7 as baits, and the Matchmaker Gold Yeast Two-Hybrid (Y2H) System (Clontech, Palo Alto, CA, USA) was used to identify FocCP1a and FocCP1b-interacting proteins. To verify the protein interactions, MaPR1B and MaPR1C without SP were introduced into pGADT7 as prey; and both of the bait and prey plasmids were co-transformed into yeast strain Y2H Gold. Transformed yeast cells were assayed for growth on synthetic

dropout (SD)/-Trp-Leu plates and SD/-Trp-Leu-His-Ade plates containing 40 μg mL^{-1} X-α-galactosidase (X-α-Gal) and 125 ng mL^{-1} aureobasidin A (ABA).

Subcellular localization and Bimolecular fluorescence complementation (BiFC)

FocCP1a and FocCP1b without SP was introduced into pEGAD and pNC-BiFC-Enn, respectively. MaPR1B and MaPR1C without SP were introduced into pNC-BiFC-Enc, respectively. Then the plasmids were transformed into the *Agrobacterium tumefaciens* GV3101. The *Agrobacterium* harboring plasmids were infiltrated into *Nicotiana benthamiana* leaves for transient expression. After two days, the leaves were sampled and observed under the confocal laser scanning microscope (Leica TCS SP8), with excitation of 488 nm argon laser, and emission wave length range of 505–525 nm.

Results

Characterization of CP1 homologes in Foc TR4

In our previous work, two predicted proteins FOIG_10415 and FOIG_07409 were detected in the secretome of SGE1 and FTF1 over-expression mutants (Zhao et al. 2020); further BlastP analysis showed that the two proteins are members of the cerato-platanin family. Phylogenetic analysis indicated that FOIG_10415 and FOIG_07409 are highly homologous to the identified CP1 in other species (Figure 1(A)); thus, the two proteins were named as FocCP1a and FocCP1b. In addition, most fungi have only one CP1, such as *S. sclerotiorum*, *B. cinerea*, and *M. oryzae* (Chen et al. 2013); but two CP1 were identified in Foc TR4, *Fusarium graminearum* and *F. oxysporum* f.sp. *lycopersici*. The open reading frames of FocCP1a and FocCP1b are both 417 bp, which encode 139 aa proteins with predicted N-terminal signal peptides (1-18 aa). FocCP1a and FocCP1b both contain well-conserved cysteine residues (C39, C78, C81, and C136), compared with the identified CP1 in other species (Figure 1(B)).

Generation of the knock-out mutants

To explore the biological functions of FocCP1 proteins, the *FocCP1a* and *FocCP1b* knock-out mutants were obtained through gene replacement strategy (Figure 2(A)). After a two-round PCR diagnosis (Figures S1 and S2) and single ascospore isolation, the correct homokaryotic knock-out mutants were obtained. As showed in Figure 2(B), the nucleotide sequence of *FocCP1a* and *FocCP1b* were completely knocked out from the genome of Foc TR4. Then the two mutants were named as ΔFocCP1a and ΔFocCP1b respectively. In addition, the double knock-out mutant of both *FocCP1a* and *FocCP1b* was constructed through deleting *FocCP1b* from the ΔFocCP1a mutant; and the double knock-out mutant was named as ΔFocCP1aΔCP1b.

FocCP1 proteins are not required for vegetative growth and conidiation

As showed in Figure 3(A), there were no difference in colony morphology and growth rate among WT, ΔFocCP1a, ΔFocCP1b, and ΔFocCP1aΔCP1b strains on complete or

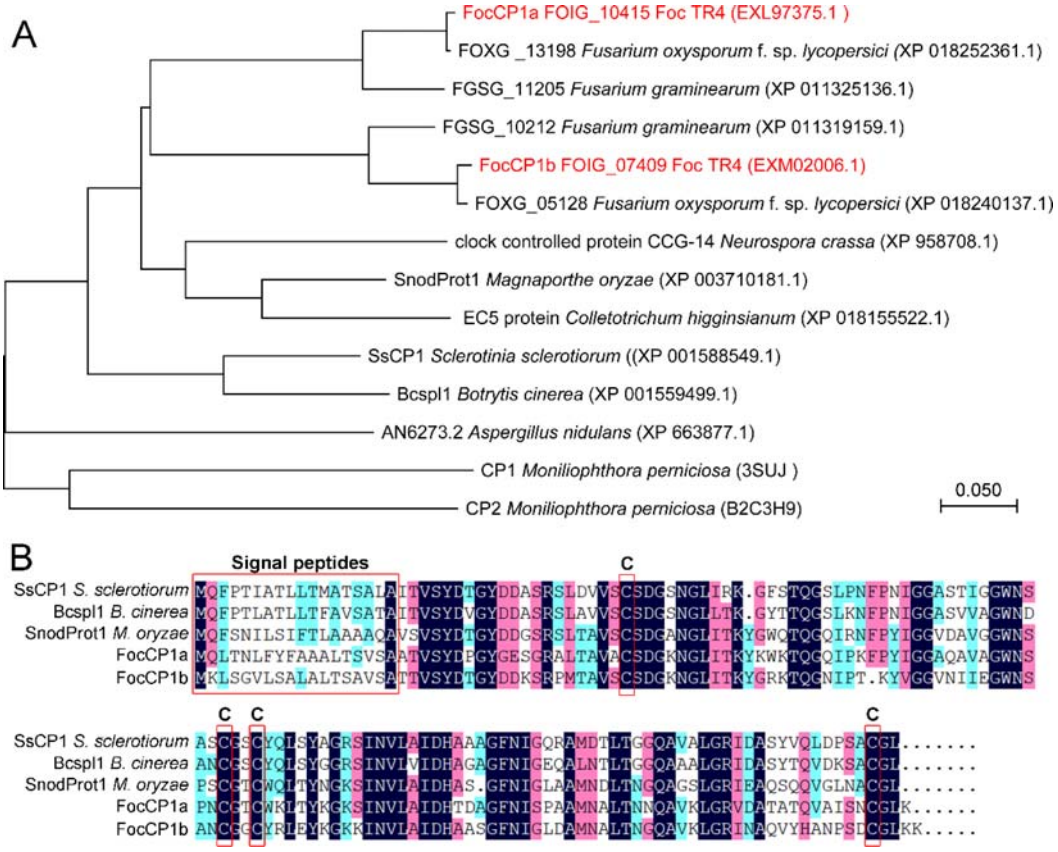

Figure 1. Bioinformatics analysis of the cerato-platanin proteins FocCP1. (A) The phylogenetic relationship of FocCP1 proteins and CPs from other fungi was determined with a neighbor-joining algorithm. (B) Comparison of conserved motifs of FocCP1 proteins with its homologues from other fungi.

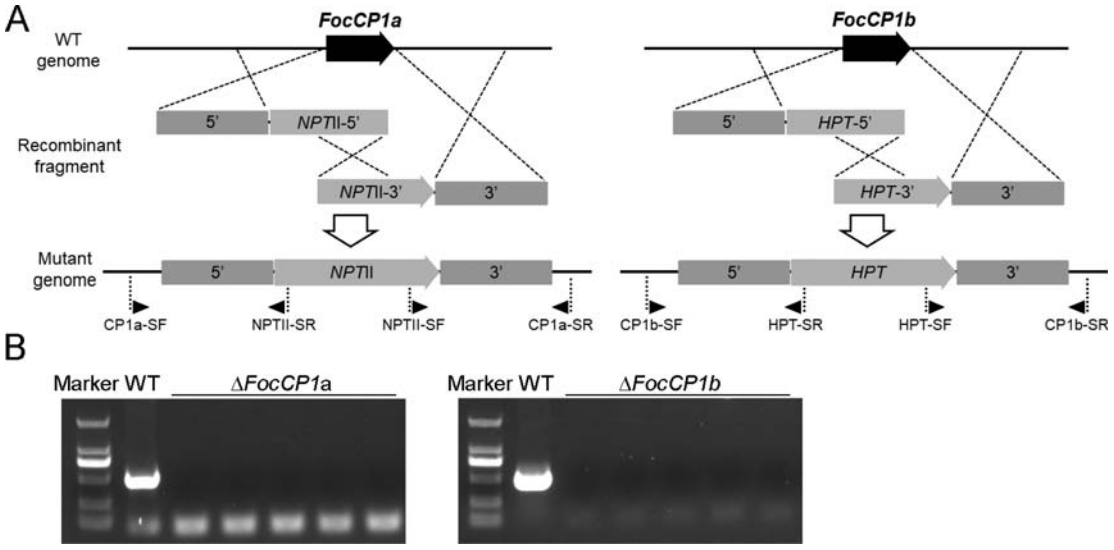

Figure 2. Construction of the gene knock-out mutants. (A) The strategies for FocCP1a and FocCP1b knock-out. Putative mutants have been screened with diagnostic primers indicated with black triangles. (B) Diagnosis for FocCP1a and FocCP1b nucleotide sequence in the genome of the mutants using PCR analysis.

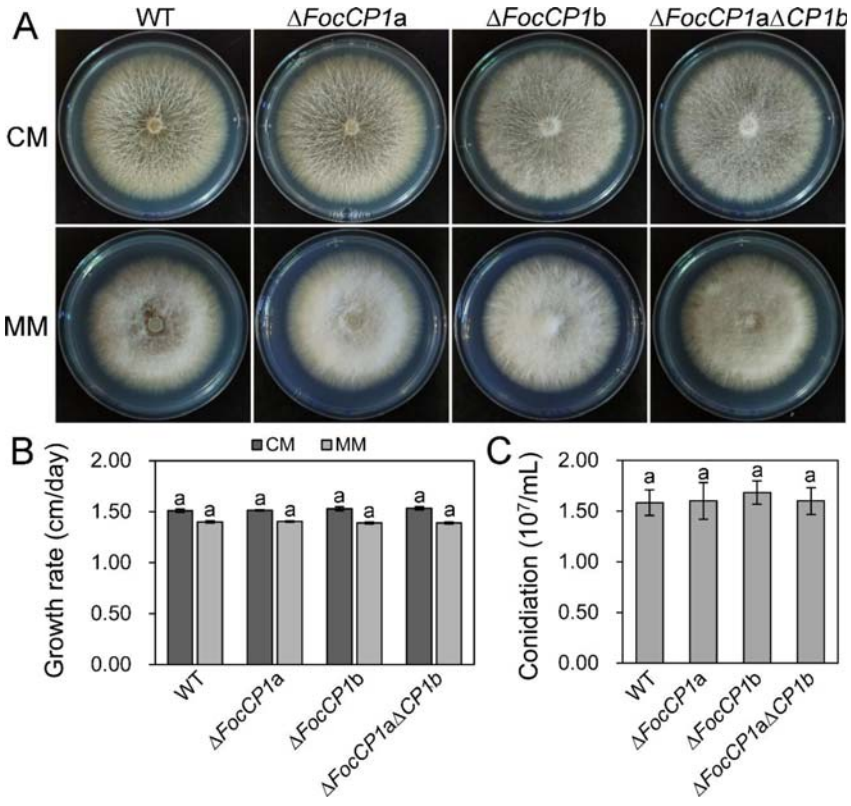

Figure 3. Growth rate and conidiation of the ΔFocCP1a, ΔFocCP1b, and ΔFocCP1aΔCP1b mutants. (A) Colony morphology of Foc strains on complete medium (CM) and minimal medium (MM) for five days. (B) Growth rate of Foc strains on CM and MM. (C) Conidiation of Foc strains. Bars represent standard deviations (SD). Columns with different letters indicate significant difference ($P < .05$).

minimal medium (Figure 3(A,B)). In addition, when cultured in liquid complete medium, the mutant strains produced similar amount of conidia as WT (Figure 3(C)). These results suggested that FocCP1 are not required for vegetative growth or conidiation in Foc TR4.

The two FocCP1 proteins play a role in the regulation of Foc TR4 virulence

Virulence of the mutants was analyzed by inoculation to Cavendish banana plantlets. After inoculation for four weeks, rhizome and pseudostem of banana plantlets were investigated for browning symptoms. As shown in Figure 4, all the knock-out mutants could infect banana plantlets. The plantlets inoculated with ΔFocCP1a or ΔFocCP1b showed similar disease symptoms as that treated with WT, with about 80% of the rhizome at disease score of 4. Whereas in the plantlets treated with the double knock-out mutant ΔFocCP1aΔCP1b, there are only 20% of the rhizome showed disease score of 3 and 0% at score of 4. These results suggested that there exist redundancy in biological functions between FocCP1a and

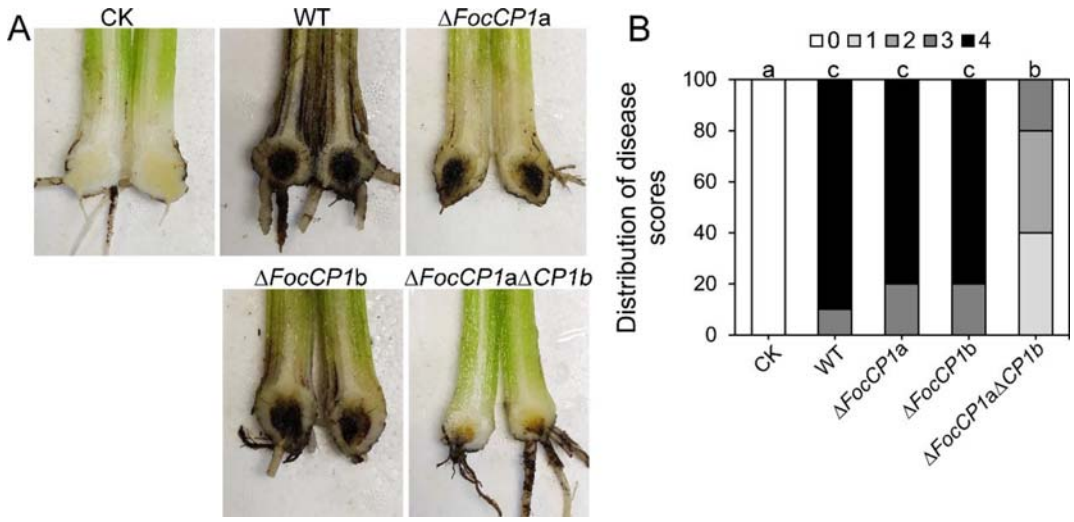

Figure 4. Virulence analysis of the ΔFocCP1a, ΔFocCP1b, and ΔFocCP1aΔCP1b mutants. (A) Disease symptoms of rhizome and pseudostem of banana plantlets after infection for four weeks. (B) Distribution of disease scores after inoculation for four weeks. Treatments with different letters indicate significant difference ($P < .05$).

FocCP1b; furthermore, the FocCP1 proteins are required for virulence of Foc TR4 to banana.

Subcellular localization of FocCP1 proteins

To analyze the subcellular localization of FocCP1, the two FocCP1-GFP fusion proteins were transiently expressed in leaves of *N. benthamiana*, and the fluorescence analysis showed that the two FocCP1 proteins were mainly distributed in the cytoplasm (Figure 5).

FocCP1 interacts with PR1

To identify the potential targets of FocCP1 proteins, FocCP1a and FocCP1b without signal peptide (SP) were introduced into pGBKT7 as baits, and the yeast two-hybrid assay was performed via screening a banana cDNA library. Two pathogenesis-related proteins MaPR1B (Ma04_p29630.1) and MaPR1C (Ma02_p15060.1) were identified to be potential interacting proteins. Sequence alignment revealed that the two PR1 proteins are highly conserved (Figure 6(A)). Then the two MaPR1 proteins without SP were introduced into pGADT7 vector and co-expressed with FocCP1-pGBKT7 to confirm the interactions between FocCP1 and MaPR1 proteins. The results suggested that both FocCP1 proteins strongly interacted with MaPR1C and slightly with MaPR1B (Figure 6(B)). Furthermore, a BiFC assay in *N. benthamiana* leaves was conducted to confirm the *in vivo* interactions through transiently co-expression of nGFP-FocCP1 and cGFP-MaPR1C in *N. benthamiana* leaves. The specific green fluorescence was detected in the apoplast of agroinfiltrated cells (Figure 6 (C)), indicating that FocCP1 interacted with MaPR1 in the plant cell apoplast. The above results suggested that FocCP1 physically interact with MaPR1.

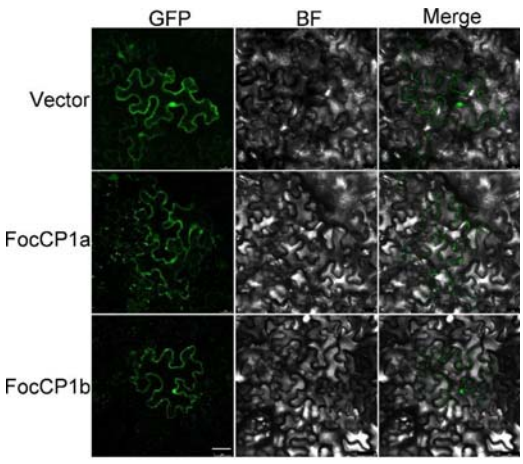

Figure 5

Figure 5. Subcellular localization of FcoCP1 proteins in *Nicotiana benthamiana* epidermal cells. Vector: cells expressing empty pEGAD; Bar: 50 μm.

Discussion

Over the past years, an increasing number of effectors have been reported in plant pathogenic fungi. While only a few effectors from Foc TR4 have been experimentally characterized (An et al. 2019; Widinugraheni et al. 2018). In our previous work, two proteins FocCP1a and FocCP1b belonging to the CP family have been identified in the secretome of Foc TR4 (Zhao et al. 2020). Phylogenetic analysis showed that the two proteins are well conserved compared with the identified CP1 in other species (Figure 1). Furthermore, most fungal pathogens have only one CP coding gene within the genome (Chen et al. 2013), such as *B. cinerea*, *S. sclerotiorum*, and *M. orzyae*; but two CP1 orthologues were identified in Foc TR4, Fol, and *F. graminearum* (Figure 1), suggesting that the two orthologues might have some special functions or are just redundancy in *Fusarium* spp.

In most of fungi, knock-out of CP1 coding genes showed no influence on growth rate, conidiation, or colony and conidial morphology *in vitro*, except for *Colletotrichum gloeosporioides*, in which knockout mutants of CgCP1 showed reduced conidiation (Frias et al. 2011; Jeong et al. 2007; Wang et al. 2016; Wang et al. 2018; Yang et al. 2009). In the present study, no significant phenotypic differences were observed among FocCP1a, ΔFocCP1b, or ΔFocCP1aΔCP1b mutants compared with the WT strain (Figure 3), suggesting that the two FocCP1 did not

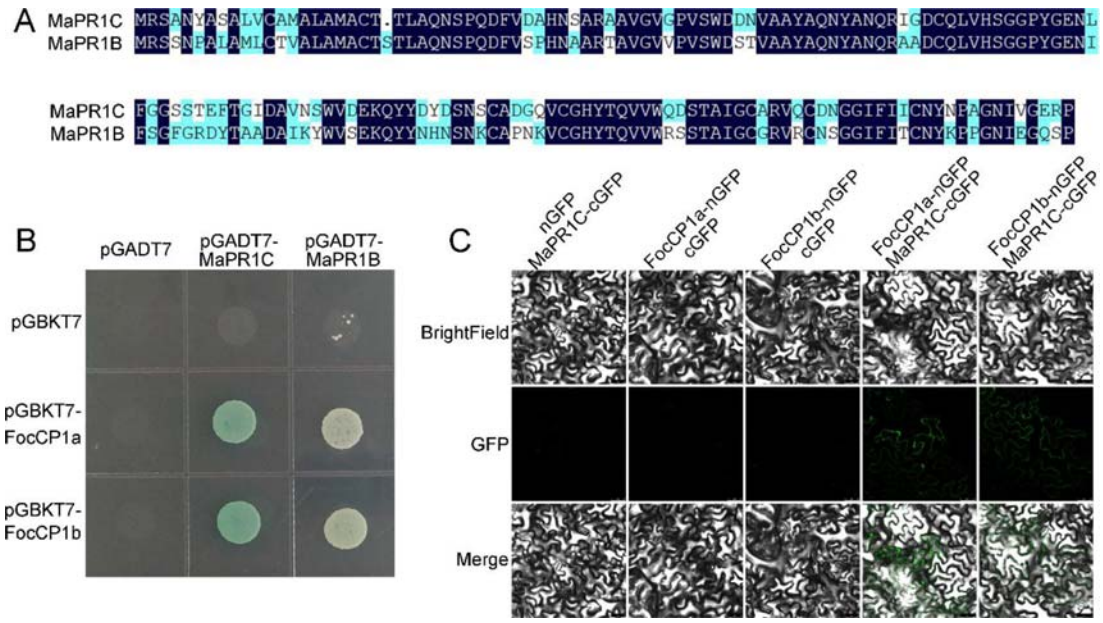

Figure 6. FocCP1 proteins interacts with MaPR1 proteins. (A) Comparison of amino acid sequence of MaPR1C and MaPR1B. (B) Yeast two-hybrid (Y2H) assays showing FocCP1 interact with MaPR1. Yeast cell were grown on SD/-Trp-Leu-His-Ade plates containing X-α-Gal ABA. (C) BiFC showing the *in vivo* interaction between FocCP1 with MaPR1C. Bar = 50 μm.

participated in vegetative growth or development in Foc TR4. In contrast, in comparison with WT strain, the virulence of ΔFocCP1aΔCP1b was significantly reduced, with a significant depression in conization of banana root systems (Figure 4). In another soil-borne pathogen *Verticillium dahlia*, knock-out of VdCP1 cause reduced virulence of the pathogen to cotton plant (Zhang et al. 2017). In several other fungal pathogens, CP1 proteins were also required for full virulence to host plant; whereas in *F. graminearum*, knock-out of the two CP coding genes showed no reduction in symptoms severity compared to the wild type strain on both soybean and wheat spikes (Quarantin et al. 2016), suggesting the diverse functions of CP1 proteins in different species.

Several experimental data proved that CP1 play important roles in the interaction between fungi and host plants. Plants have evolved receptors to sensing fungal chitin and then induce immunity (Chisholm et al. 2006; Radutoiu et al. 2007). In response, some fungal CP proteins could bind to chitin and help the microbe escaping from being detected by plant and successful colonization (Baccelli et al. 2012; **de Oliveira et al. 2011**; Frischmann et al. 2013); while FgCP proteins from *F. graminearum* could bind to a soluble cellulose derivative (Quarantin et al. 2016). In addition, CP1 homologues could induce cell death of host plant and facilitate colonization of the necrotrophic and hemibiotrophic pathogens *B. cinerea*, *S. sclerotiorum*, *M. oryzae*, and *C. gloeosporioides* (Frias et al. 2011; Jeong et al. 2007; Wang et al. 2016, 2018; Yang et al. 2009). In the recent report, the purified recombinant FocCP1a protein could induce a series of defense reactions in tobacco, including accumulation of reactive oxygen species (ROS), formation of necrotic reaction, deposition of callose, expression of defense-related genes, and accumulation of salicylic acid (SA) and jasmonic acid (JA) (Li et al. 2019), and *V. dahlia* VdCP1 showed similar effect on cotton plant (Zhang et al. 2017).

However, the molecular mechanism of FocCP1 in regulation of plant–pathogen interactions remain elusive. So the Y2H analysis screening a banana cDNA library was conducted and two PR1 proteins, MaPR1B and MaPR1C, were identified. Further confirmation and BiFC tests suggested that FocCP1 proteins interact with MaPR1B and MaPR1C proteins (Figure 6). PR1s are widely distributed in plants and are involved in defense against fungal pathogens (Niderman et al. 1995; Rauscher et al. 1999); furthermore, the expression of PR1 is induced by SA (salicylic acid) and associated with SAR (system acquired resistance) (van Loon et al. 2006). Exogenous application of salicylic acid could increase the resistance of banana, tomato, and *Arabidopsis* to the *F. oxysporum* (Edgar et al. 2006; Mandal et al. 2009; Wang et al. 2015). AtPR1 is known to localize in the apoplast; in the present study, the FocCP1 proteins were found mainly distributed in the cytoplasm of *N. benthamiana* (Figure 5). Therefore, we speculate FocCP1 could interact with MaPR1 and inhibit the potential antifungal activity of PR1.

Taking together, the effectors FocCP1 are redundancy in the regulation of full virulence of Foc TR4 to banana plants; furthermore, the two FocCP1 might inhibit the antifungal activity of MaPR1 proteins by directly interacting with them.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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