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Engineering a peptide aptamer to target calmodulin for the inhibition of *Magnaporthe oryzae*

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ABSTRACT

To develop an antimicrobial agent for preventing the devasting damage caused by rice blast, a novel peptide aptamer was identified to interact with calmodulin (CaM) for the inhibition of the spore development in the pathogen *Magnaporthe oryzae*. A peptide aptamer designated as SNP-D4, consisted of the scaffold protein *Staphylococcus aureus* nuclease (SN) and an exposed surface loop of 16 random amino acids, was screened from the constructed peptide aptamer libraries by bacterial two-hybrid system using CaM of *M. oryzae* as the bait. The preliminary inhibition in the sporulation development was observed after treating with the crude extracts expressing SNP-D4. The inhibition efficacies of the purified SNP-D4 were quantified at the stages of conidial germination, germ tube elongation, and appressorium formation in *M. oryzae*. The binding affinity analysis revealed that SNP-D4 interacted with CaM at a dissociation constant (K_d) of about 20 μM. Moreover, the N-terminus of CaM was identified as the key binding region.

1. Introduction

Since rice blast caused by *Magnaporthe oryzae* (*M. oryzae*) incurs serious diseases on economic crops and caused significant yield reduction and food insecurity, the biocontrol of the phytopathogenic *M. oryzae* has been of great concern (Yan and Talbot, 2016). *M. oryzae* is a type of heterozygous haploid ascomycete and propagates asexually to produce conidia. The initial infection process involves spore adhesion, spore germination, germ tube growth, and appressorium formation, wherein the latter two stages are crucial for rice blast infection (Hamer and Talbot, 1998). As Ca^{2+} signaling pathway is implicated in these crucial processes during fungal infections, the regulators for this pathway have been considered as specific targets for the inhibition of *M. oryzae* (Lee and Lee, 1998). CaM is widely presented in various development stages, particularly in early sporulation development of *M. oryzae*. And it acts as an important regulator in the Ca^{2+} signaling pathway and modulates the viability of pathogens in the host (Rho et al., 2009). Systematic functional analysis shows that at least 35 out of 37 known calcium-related signaling proteins are involved in sporulation (Nguyen et al., 2008). Therefore, CaM constitutes a potential target for drugs to inhibit and prevent *M. oryzae*, and provides an alternative way to explore the effective prevention strategies against the phytopathogenic infection.

Chemical fungicides have been widely applied to control pathogenic fungi, and cause seriously adverse effects including environmental pollution and pathogenic resistance (Cools and Hammond-Kosack, 2012). Peptide aptamers refers to short peptides that possess specific and high affinity with the targets, which consists of a biologically inactive scaffold protein and variable peptides immobilized on the scaffold by the end (Mascini et al., 2011). The peptide aptamers can be transferred into crops and confers broad pathogen resistance by interacting with specific target in the pathogen (Fernández Acero et al., 2011). When expressing peptide aptamers that bind strongly with the replication initiator proteins Rep/AL1, the transgenic tomatoes display the reduced symptoms and decreased viral DNA loads after the infection of tomato mottle virus (Reyes et al., 2013). Furthermore, cell-penetrating peptides (CPPs) can penetrate into plant cells and provide an alternative to traditional transgenic strategy (Colombo et al., 2015). All of the above accelerate the application of peptide

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aptamers as a biological agent which can be directly sprayed on the surface of the plants (Fernández Acero et al., 2011). Accordingly, we screened a peptide aptamer SNP-D4 from a peptide aptamer library using CaM of *M. oryzae* as the bait. The application of peptide aptamer is a rational design, creating an accessible method that will allow rapid and effortless biological control of rice blast.

2. Materials and methods

2.1. Bacterial strains, growth conditions, and plasmids

M. oryzae was grown on Potato Dextrose Agar (PDA) or cultured with aerobic shaking in Potato Dextrose Broth (PDB) at 30 °C. The bacterial strains were grown on LB Agar or cultured with shaking in LB Broth at 37 °C. The used primers are summarized in Table 1. The plasmids pET-*28a* and its derivatives endowing with *trc* promoters were induced with the final concentration of 1 μM IPTG. The plasmid pBT was supplied in the BacterioMatch® II Two-Hybrid System Vector Kit, and used as a bait vector bearing the fusion expressions of bacteriophage λ repressor protein (λcI) and CaM. The target vector pTRG expresses the fusion of the peptide aptamer and α subunit of RNA.

2.2. Construction of the peptide aptamer library

Genomic DNA of *Staphylococcus aureus* was extracted and the scaffold protein nuclease (SN) was amplified using primer pairs of 5SNA/3SNA and 5SNB/3SNB as shown in Table 1. The PCR product was digested and ligated to vector pTRG, producing the recombinant vector pTRG-SN. Then the random nucleotide fragment of "5′-GGTGGTNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNSNNS-NNSNNSNNSGGTGGT-3′" was synthesized, wherein N and S representing any of A/G/T/C or G/C, respectively. The single-stranded DNA was converted to double-stranded DNA, and inserted into pTRG-SN. The resulting vectors pTRG-SNPs expressed both scaffold protein nuclease and an exposed surface loop of 16 random amino acids. The vector pTRG-SNPs were transformed into *Escherichia coli* XL1-Blue MR to construct the aptamer library. The random clones were titrated as the library capacity of about $1.5–2.0 \times 10^7$ cfu/ml.

2.3. Bacterial two-hybrid system

The bacterial two-hybrid system was performed according to the protocol of BacterioMatch II two-hybrid system. Briefly, the bait plasmid pBT-*CaM* was co-transformed with pTRG-*SNPs* into *E. coli* XL1-Blue MR. The positive colonies were selected on the 5 μM 3-AT media, and recovered for further examination on the media containing 5 μM 3-AT and streptomycin. To eliminate pBT-*CaM*, the resulting clones were streaked onto 12.5 μg/ml tetracycline LB agar, and the pTRG derivatives were extracted for DNA sequencing.

To evaluate the key regions of CaM interacting with peptide aptamer, CaM were truncated, and co-transformed successively with peptide aptamer for bacterial two-hybrid analysis.

Table 1
Primers in this study.

Primers	Sequnce (5′–3′)	Enzyme
5SNA	GGATCCGCGGCCGCAATGGGTTACCCATACGACGTTC	*Bam* HI
3SNA	GTACTT AGATCTGGCCTTTCTTAAGGAGAATTCTG	*Bgl* II
5SNB	AAGGCCAGATCTAAGTACGGTCCAGAAGCTTC	*Bgl* II
3SNB	GATCTCACTAGTTTAGTGGTGGTGGTGGTGGTCG	*Sca* I
CaM-F	ATCGAATTCGATGGCTGATTCGCTTACCGAAGA	*EcoR* I
CaM-R	CCCTCGAGTTACTTTTGCATCATGAGCTGGAC	*Nco* I
SNP-D4-F	CATGCCATGGATGGGTTACCCATACGACGT	*Xho* I
SNP-D4-R	CCGCTCGAGGTCGATGTCAACTTGACCAG	*EcoR* I
CaM△C73-F	ATCGAATTCGATGGCTGATTCGCTTACCGAAGA	*EcoR* I
CaM△C73-R	CCCTCGAGTTATTTCCTGGCCATCATGGTGAG	*Xho* I
CaM△N76-F	ATCGAATTCGATGAAGGATACCGACTCGGAGGA	*EcoR* I
CaM△N76-R	CCCTCGAGTTACTTTTGCATCATGAGCTGGAC	*Xho* I

2.4. Protein purification

The plasmids pET-28a and its derivatives were transformed into *E. coli* BL21. The recombinant strains were induced with 100 mM IPTG and then harvested by centrifugation at 5000 rpm for 10 min prior to be suspended in 10 μM PBS (pH 7.4). Lysate was loaded onto a Ni-NTA column after sonication and the column was washed with 15 ml of 10 μM PBS. The miscellaneous proteins were removed by gradually elevated concentrations of imidazole (20–100 μM) and the target protein was eluted eventually with elution buffer (1.5 μM NaH_2PO_4, 8 μM Na_2HPO_4, 150 μM NaCl, 250 μM imidazole, pH 7.4). The purified protein was desalted in dialysis buffer (1.5 μM NaH_2PO_4, 8 μM Na_2HPO_4, 150 μM NaCl, 100 μM KCl, 5 μM β-mercaptoethanol, 50 % glycerin pH 7.4) at 4 °C. After the molecular weight and purity of fusion proteins were detected by 12 % SDS-PAGE, the concentration was determined by BCA assay.

2.5. Enzyme linked immunosorbent assary (ELISA)

The total of 100 μl His-tagged CaM (0.02, 0.05, and 0.1 μg/ml, respectively) were coated on the 96-well microplates, and incubated with PBS-T buffer (0.02 M PBS supplemented with 0.05 % Tween 20) at 4 °C overnight. After blocking with 5 % nonfat dry milk in the following day, the wells were washed three times with PBS-T, and incubated with 100 μl rabbit antibody (1:1000 in PBST, Thermo Fisher Scientific) against SN at 37 °C for 90 min. Subsequently 100 μl HRP-conjugated anti-rabbit goat antibody (1:1000 in PBST, Thermo Fisher Scientific) was added and cultured for 90 min, followed by the additions of 100 μl TMB substrate for 30 min and 100 μl TMB termination buffer for quenching the reaction. The absorbance was measured using Microplate Readers, and the dissociation constants K_d were analyzed by virtue of GraphPad Prism version 6.0.

2.6. Microscopic observation of the effect of peptide aptamer on infection process of M. oryzae

The effect of peptide aptamer on the morphology of *M. oryzae* was evaluated on the surface of glass slide. Conidia were collected from 6-d cultures, and mixed with cell lysate or various concentrations of purified peptide aptamer with the final concentration of conidial suspension as 5×10^5 cell/ml. The samples were pipetted on glass slides and incubated at 28 °C for 0, 3, 6 and 12 h. The fungi undergoing conidial germination, germ tube elongation and appressorium formation were counted with three independent experiments per treatment. The treatments with both PBS buffer and scaffold SN were chosen as the negative controls, and tricyclazole was selected as the positive concurrently. The percentages of conidial germination, germ tube elongation and appressorium formation were estimated using one-way ANOVA procedure of SPSS Statistics 17.0.

3. Results

3.1. Identification of peptide aptamers for the inhibition of M. oryzae

CaM was constructed as the bait plasmid pBT-*CaM* to select a specific peptide aptamer by bacterial two-hybrid system. First, the

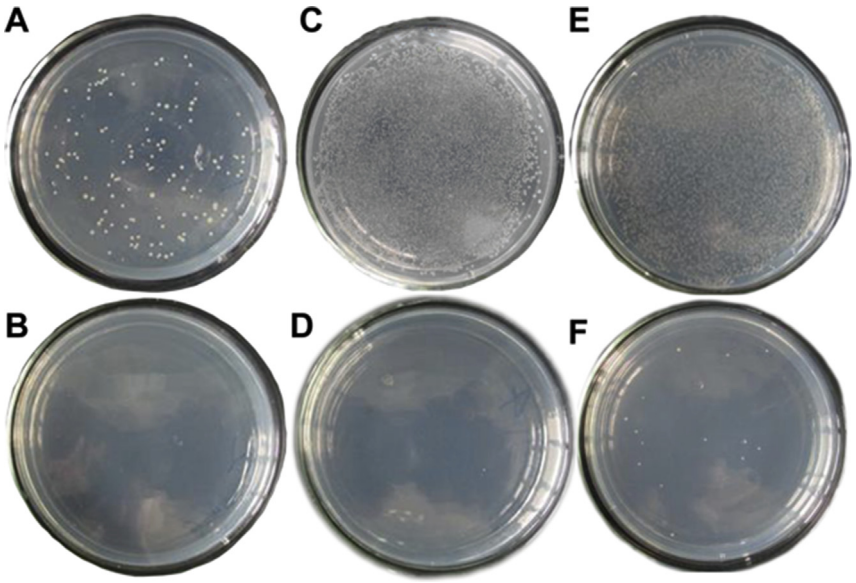

Fig. 1. Identification of peptide aptamers which interact with CaM of *M. oryzae* by bacterial two-hybrid. The self-activation assay was validated by co-transformation of pBT-*CaM* and pTRG vector on plates with (A) no 3-AT and (B) 5 mM 3-AT, and of pBT-*CaM* and pTRG-*SN* containing scaffold protein SN on plates with (C) no 3-AT and (D) 5 mM 3-AT. The potential pepetide aptamers were screened by **c**o-transformation of pBT-*CaM* and pTRG-*SNPs* carrying pepetide aptamer library on plates with (E) no 3-AT and (F) 5 mM 3-AT.

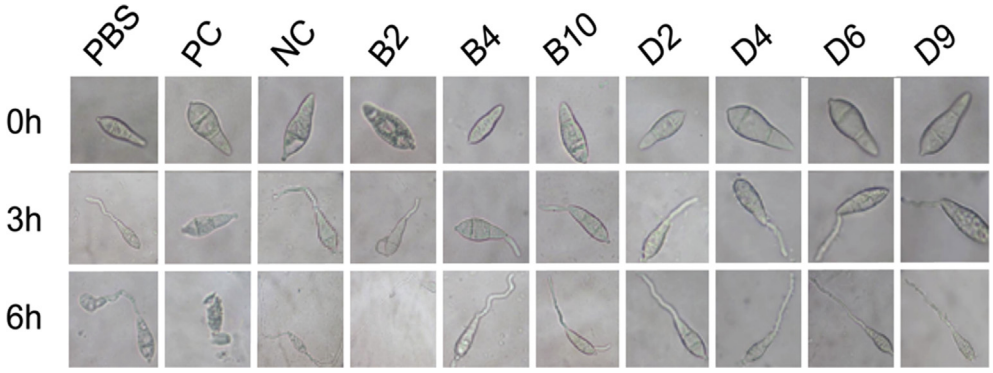

Fig. 2. Effects of the candidate peptide aptamers on the infection process of *M. oryzae* detected by microscopy observation (40×). PBS, the treatment with PBS buffer as the blank control; PC, the treatment with 0.2 % tricyclazole solution as the positive control; NC, the treatments with the crude extract containing SN protein as the negative control; B2, B4, B10, D2, D4, D6, and D9, the treatments with the corresponding candidate peptide aptamers.

Table 2
The nucleotide sequence of the candidate peptide aptamer.

Candidate	Nucleotide sequence
B2 (48 bp)	**CCGGGGCCCGAGGGGGACATGGAGGAGCGGAACACGTCGTAGTGCAAC**
B4 (49 bp)	TGTGGAGTTCGCCGCCCGGTAGCCGGTCGCCTAGGGCATGGTGGGGATG
B10 (49 bp)	CCCGGGGTGCACGGTCCGCTAGAGCAGCAGCGGGAGGCGGGCGATGAGG
D2 (49 bp)	TGTGGAGTTCGCCGCCCGGTAGCCGGTCGCCTAGGGCATGGTGGGGATG
D4 (48 bp)	**GTCACCTTCCTCGTGAACACCTACCCGAACGGGGTCCAGAGCAGGGCC**
D6 (48 bp)	**ATGCGGGGGTGCGGGTCGTAGTGGTGCAAGGACTAGCCCGCCGGGGTC**
D9 (53 bp)	TGTGCGGCGGGGGGGGCGTCCGGCGTTCTACGTCTGCGGGCAGGGCCTGGCCC

constructed bait plasmid was verified not to induce a self-activation in the system (Fig. 1B and D). CaM itself could neither produce positive colonies by co-transformed with empty vector pTRG on 3-AT selective media, nor interact with the scaffold protein SN. Then, bacterial two-hybrid assays were conducted between the bait plasmid pBT-*CaM* and the peptide aptamer library (pTRG-SNPs),

Table 3
The amino acid sequence of the candidate peptide aptamer.

Candidate	Amino acid sequence
B2	PGPEGDMEERNTS*CN
D4	**VTFLVNTYPNGVQSRA**
D6	MRGCGS*WCKD*PAGV

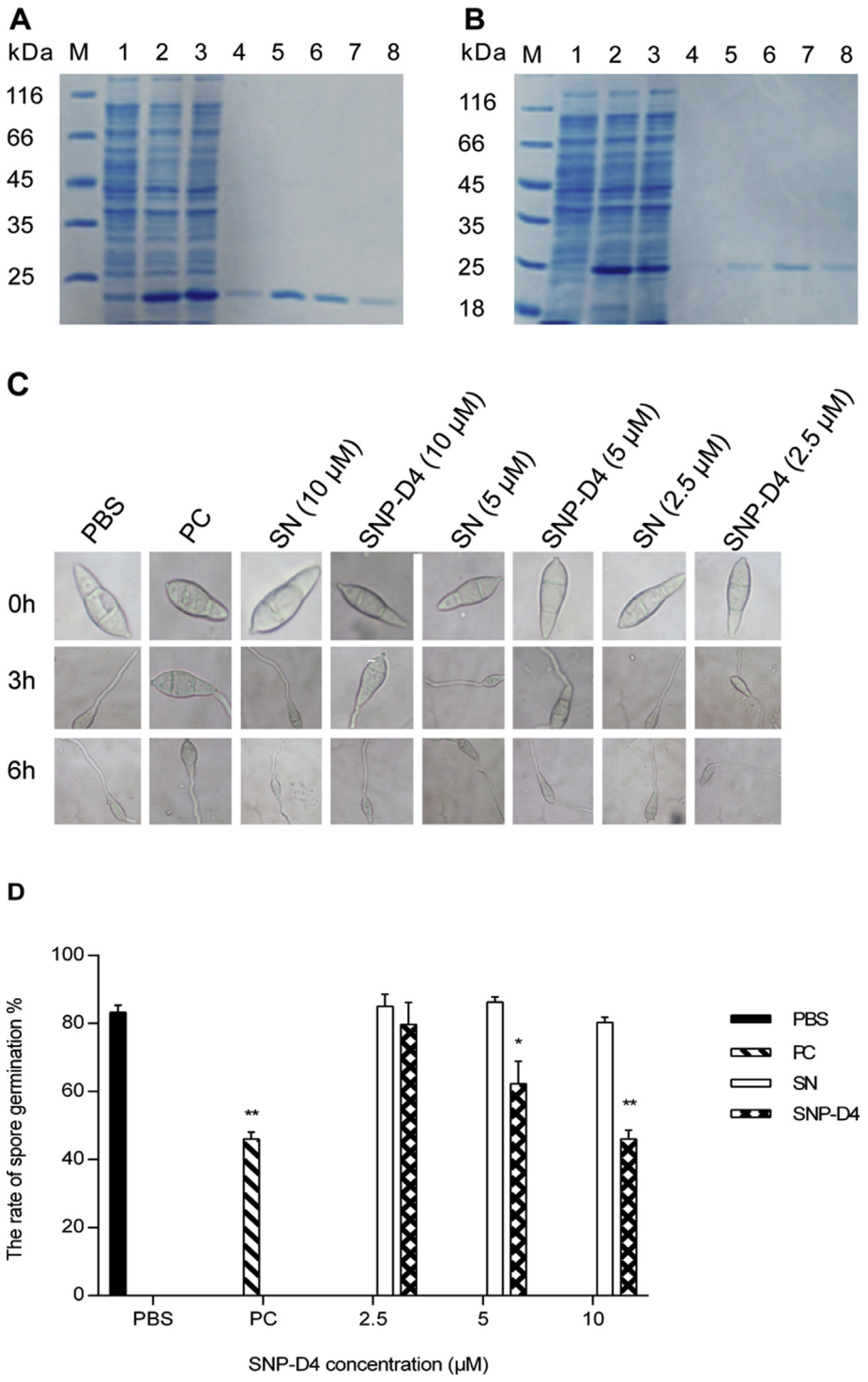

Fig. 3. Inhibition of the infection process of *M. oryzae* using the purified peptide aptamer SNP-D4. The purified (A) SNP-D4 and (B) SNP were detected by 12 % SDS-PAGE gel. Lane M, molecular mass marker; lanes 1–8, the eluted fractions with 20, 50, 100, 150, 200, 250, 300, 500 mM imidazole. (C) The inhibition of the sporulation development was detected by microscopic observation (40×). The rates of fungi undergoing spore germination (D) and appressorium formation (F), as well as the length of germ tube (E) were measured after different treatments. PBS, the treatment with PBS as the blank control; PC, the treatment with 0.2 % tricyclazole solution as the positive control; SN, the treatments with the indicated concentrations of the purified SN protein; SNP-D4, the treatments with the indicated concentrations of the purified SNP-D4.

and totally 34 candidates which specifically interacted with CaM were selected for identification (Fig. 1E and F).

In order to evaluate the efficacies of the interested peptide aptamers, the candidate strains containing the expressed peptide aptamers alone were induced with IPTG, and the crude extracts were tested for the capability to inhibit the sporulation development of *M. oryzae*. The results showed that, the spore germination was evidently inhibited after the incubation with the

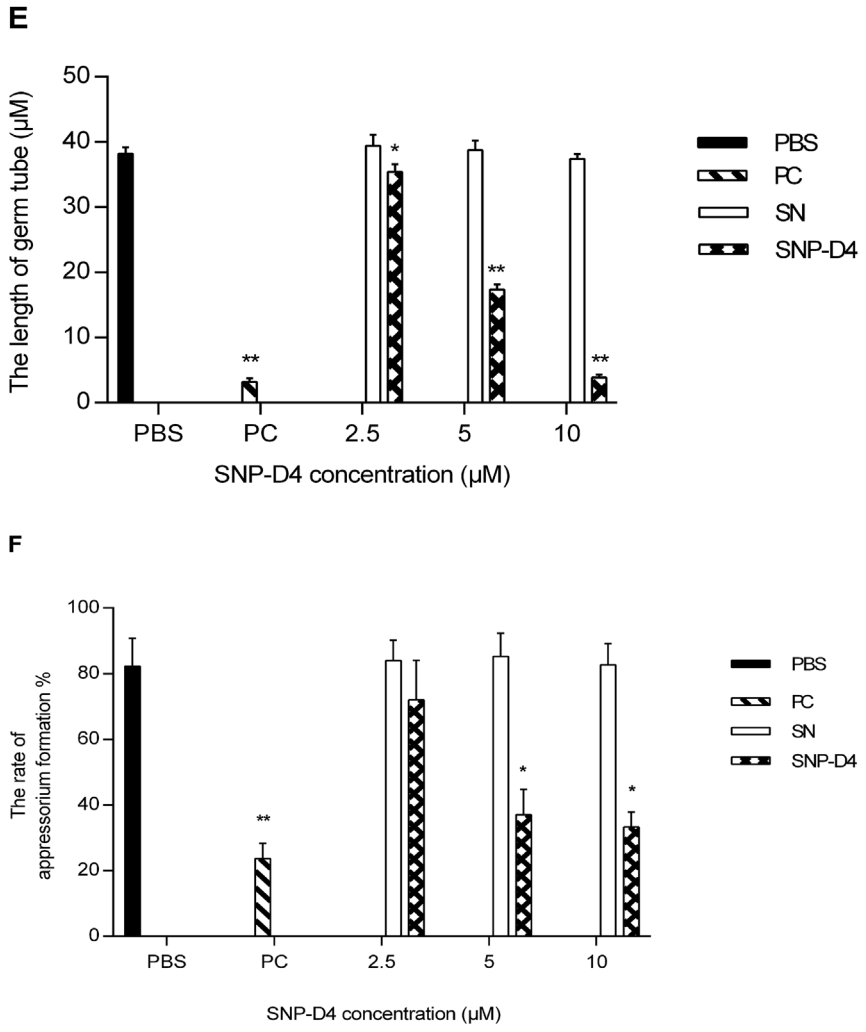

Fig. 3. (*continued*).

candidates B2, B4, B10, D2, D4, D6 and D9 for 3 h. Similarly, the elongation of germ tube was suppressed after the incubation for 6 h (Fig. 2). The nucleic and amino acid sequences of the candidates are shown in Tables 2 and 3, respectively. The candidates B4, B10, D2, and D9 were discarded due to their incomplete set of triple codons, which led to the frameshift mutation in the followed region of scaffold SN (Table 2). Also B2 and D6 were rejected because the stop codon was introduced in the encoding region, which resulted in the early termination of the translation (Table 3). Eventually, a novel peptide aptamer named as SNP-D4, whose amino acid sequences consisted of "VTFLVNTYPNGVQSRA", was identified to inhibit the sporulation development of *M. oryzae* by interacting with CaM.

3.2. *Peptide aptamer SNP-D4 inhibits the infection process of* M. oryzae

To confirm the inhibitory activity to the sporulation development of *M. oryzae*, SNP-D4 and SN were induced by IPTG, and purified with Ni-Sepharose affinity column, and enriched by dialysis. Both SNP-D4 and SNP were highly purified with appropriate molecular weights of about 18 kD and 21 kD, respectively (Fig. 3A and B). The different concentrations of SNP-D4 and SN were incubated with 5×10^5 cell/ml of conidial suspension, and the sporulation development was observed under optical microscope after incubation for 0, 3, and 6 h (Fig. 3C). Meanwhile, conidial suspensions treated with 0.01 % tricyclazole and PBS were used as the positive and negative controls separately. The inhibition of spore germination was positively correlated with the concentrations of SNP-D4. The concentration of 10 μM SNP-D4 exhibited similar effect as that achieved by 0.01 % tricyclazole, while 10 μM of SN displayed little suppression at 3 h (Fig. 3C). Likewise, the elongations of germ tube were allowed in SN and PBS at 6 h, while the various degrees of inhibition were observed in 0.01 % tricyclazole and SNP-D4 (Fig. 3C).

The rates of conidial germination, germ tube elongation, and appressorium formation were calculated after incubation for 3, 6, and 12 h, individually. Compared with the corresponding concentrations of SN, treating with 5 and 10 μM SNP-D4 for 3 h suppressed the rates of spore germination significantly ($p < 0.05$) and extremely significantly ($p < 0.01$), respectively (Fig. 3D). After incubation for 6 h, the lengths of germ tube were inhibited significantly ($p < 0.05$) when treating with 2.5 μM SNP-D4; and were suppressed extreme significantly ($p < 0.01$) when treating with 5 or 10 μM SNP-D4, as compared with SN (Fig. 3E). After incubation for 12 h, appressorium formation was significantly inhibited ($p < 0.05$) when treating with 5 or 10 μM SNP-D4 (Fig. 3F). Taken together, these results demonstrated that SNP-D4 inhibited the infection process of *M. oryzae* in a concentration-dependent manner.

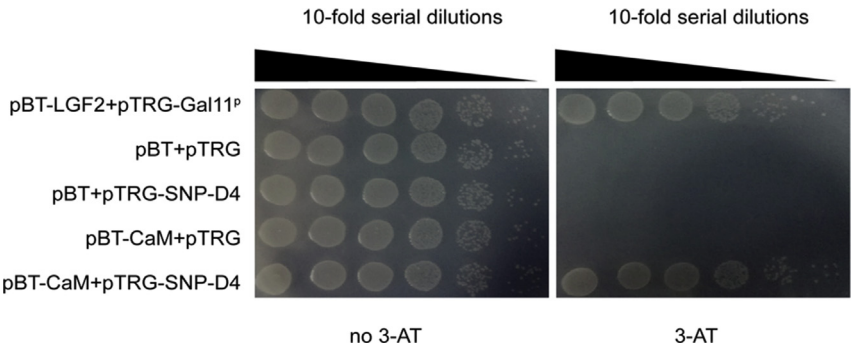

Fig. 4. Confirmation of the interaction between CaM and SNP-D4 by bacterial two-hybrid assay.

3.3. SNP-D4 interacts with N-terminus of CaM

Bacterial two-hybridization was performed to verify the interaction between SNP-D4 and CaM. The results showed that the colonies co-expressing both SNP-D4 and CaM could grow on 3-AT selective media, while the transformants expressing of empty vector, SNP-D4 or CaM alone did not (Fig. 4). Meanwhile the interaction between LGF2 and Gal11^{P} was chosen as the positive control (Fig. 4).

Then ELISA assay was further conducted to measure the affinity between SNP-D4 and CaM. First, His-tagged CaM was induced, purified and enriched, appearing as a single band with the molecular weights of about 20 kD in SDS-PAGE gel (Fig. 5A). Then, 20, 50, and 100 μg/ml CaM protein were coated and incubated with various concentrations of SNP-D4. The results showed that the binding affinity between SNP-D4 and CaM was enhanced with the increased concentrations of SNP-D4, and the saturation was reached when 1 μM SNP-D4 was added. The K_d values were calculated as 19.02, 23.03, and 20.55 μM when CaM protein was coated at 20, 50, and 100 μg/ml respectively, implying that the binding affinities between CaM and SNP were obtained at the magnitude of micromolar, and the elevated concentration of CaM did not change so much (Fig. 5B).

To figure out the key domain of CaM that mediated the interaction with SNP-D4, the full length of CaM was truncated as two parts. The structure prediction revealed that CaM contained dual EF-hand domains spanning from 12 to 74 and 85–147 amino acids. Each EF-hand domain composed of eight Ca^{2+} binding sites and was balanced on either side (Fig. 6A). The N- and C-terminal CaM were truncated and named as CaM△C73 and CaM△N76, respectively. The tertiary structure prediction showed that both truncations maintained the original structures, indicating that deletion did not destroy the tertiary structure (Fig. 6B and C). Bacterial two-hybrid assay was performed using CaM△N76 or CaM△C73 as the bait and SNP-D4 as the target. SNP-D4 could bind specially to the truncation CaM△C73, suggesting that N-terminal CaM ranging from 1 to 76 amino acid was competent for interaction with SNP-D4 (Fig. 6D).

4. Discussion

M. oryzae is the most important fungal pathogen with a high degree of host specificity, which caused threatening rice blast and great loss in agricultural economy (Islam et al., 2016). Accordingly, efficient inhibitors against *M. oryzae* are urgently needed for the sustainable development of rice agriculture, and previous studies have sought to identify such inhibitors. A sesterterpene sulfate is isolated from the tropical sponge *Hippospongia* spp as an inhibitor against isocitrate lyase (ICL). It reduces the appressorium formation of *M. oryzae*, but does not inhibit the spore germination (Shin et al., 2007). Bromophenols is isolated from the red alga *Odonthalia corymbifera*, and targets with the key enzyme isocitrate lyase (ICL) for the inhibition of appressorium (Lee et al., 2007). The cell lysate of *Staphylococcus* sp. strain LZ16 exhibits strong inhibition activities against *M. oryzae* (Yu et al., 2013). As appressorium formation is the preliminary basis for *M. oryzae* infection (Yan and Talbot, 2016), the protein CaM is ubiquitously expressed in the spore germination and appressorium formation of *M. oryzae* (Zelter et al., 2004). When the spore density of *M. oryzae* is increased or exogenous inhibitor is added, the formation of appressorium is suppressed and the expression of *CaM* is significantly decreased;

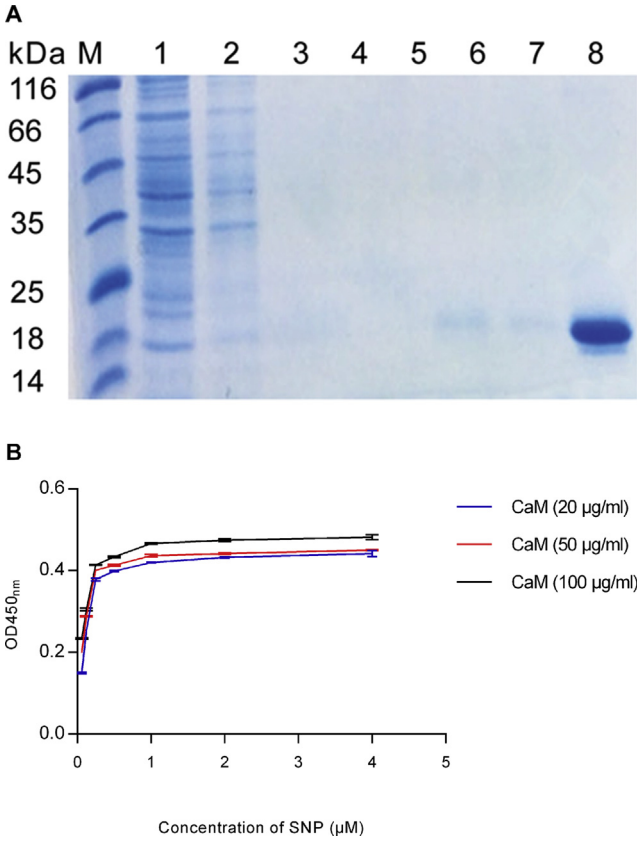

Fig. 5. Binding affinity between SNP-D4 and CaM using ELISA. (A) The purification of recombinant CaM. Lane M, the molecular mass markers; lanes 1–8, the eluted fractions with 20, 50, 100, 150, 200, 250, 300, 500 mM imidazole. (B) The measurement of dissociation constant between CaM and SNP-D4 protein.

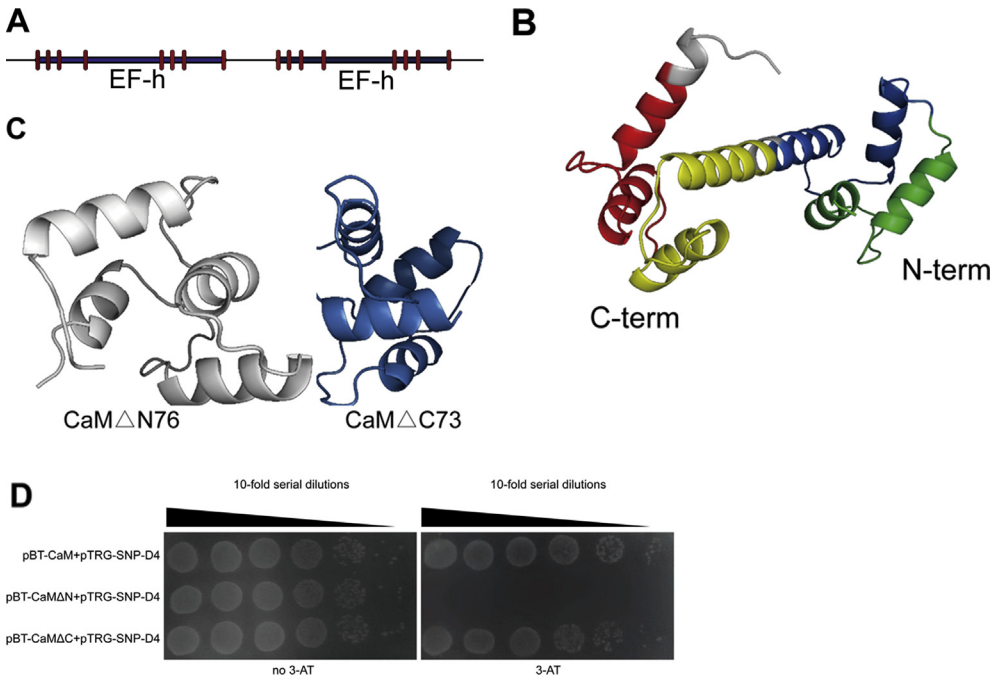

Fig. 6. Identification of the key interaction region between SNP-D4 and CaM. (A) The predicted structure of CaM with dual EF-hand domains, each of which contained eight Ca^{2+} binding sites. The horizontal blue bands represented two EF-hand domains, and the red bars indicated the Ca^{2+} binding sites. The predicted tertiary structures of full length CaM (B), and the C- and N-terminal truncations (C). Identification of the key domain of CaM interacting with SNP-D4 by bacterial two-hybrid assay (D).

inversely, when the spore concentration is reduced or the wax on the surface of plants is added, the inhibition of spore germination is relieved and the expression of *CaM* is returned to normal (Liu and Kolattukudy, 1999). RNA interference of *CaM* gene decreases the rates of spore germination and appressorium formation seriously, indicating that CaM plays an important role in sporulation development of *M. oryzae* (Kim et al., 2018). Therefore, CaM constitutes a beneficial target for inhibitors against *M. oryzae*, and the potential antagonists against calmodulin provides alternative strategies for prevention and treatment of rice blast.

The peptide aptamer screening system is documented as an efficient shortcut to discover functional short peptides (Butz et al., 2001).The synthetic peptide PAF104 reduces the appressorium formation in a concentration-dependent manner; It effects slightly on appressorium formation at a concentration of 5 μM after 6 h incubation, but achieves more than 50 % and 80 % inhibition rates at 10 μM and 30 μM, respectively (Reyes et al., 2013). In our study, the two-hybrid system was performed to screen peptide aptamers that interact specifically with CaM, and a novle peptide aptamer SNP-D4 was identified. Moreover, the inhibition assays of spore germination and appressorium formation are commonly used to estimate the antifungal effects of compounds or pepetides (Rebollar et al., 2013; Yu et al., 2013). SNP-D4 had a significantly inhibitory effect on the development of *M. oryzae* spores, specifically by reducing 38 % and 85 % of the germination rate and the length of germ. Likewise, SNP-D4 achieved a similar inhibition effect on the appressorium formation as to that of PAF104 at 10 μM concentration (Reyes et al., 2013).

In summary, the peptide aptamer SNP-D4 was identified to develop as a promising antagonist of CaM.

It could not only use for the study of Ca^{2+} signaling pathway, but also for the control of fungal infection caused by *M. oryzae*.

Acknowledgments

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