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Research Paper

An engineered multidomain fungicidal peptide against plant fungal pathogens

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Abstract: Fungal pathogens represent major problems for human health and agriculture. As eukaryotic organisms, fungi share some important features with mammalian cells. Therefore, current anti-fungal antibiotics often can not distinguish between fungi and mammalian cells, resulting in serious side effects in mammalian cells. Accordingly, there is strong impetus to develop antifungal alternatives that are both safe and effective. The E1 family of colicin are channel-forming bacteriocins produced by *Escherichia coli*, which are bactericidal only to *E. coli* and related species. To target the channel-forming domain of colicin to fungal cell membrane, we engineered a sexual mating pheromone of *Candida albicans*, α-factor pheromone to colicin Ia. A peptide was constructed consisting of an α mating pheromone of *C. albicans* fused to the channel-forming domain of colicin Ia to create a new fusion protein, pheromonicin-CA (PMC-CA). Indirect immunolabeling showed that the PMC-CA bound to fungal cells and inhibited growth in the laboratory and field. In the field, the protective activity of pheromonicin against rice blast disease was significantly greater, on a molar basis, than that of triazoles, tricyclazole or isoprothiolane. These results suggest that fusion peptides may be of value as fungicidal agents under agricultural conditions.

Key words: bactericidal protein; mating pheromone; fusion fungicidal peptide

一种对抗植物病原真菌的工程化多结构域抗真菌多肽

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摘 要：真菌病原体已成为危害人类健康和农业的主要难题。作为一类真核细胞，真菌某些重要的生物特性与哺乳类动物的细胞雷同，以致现有的抗真菌抗生素往往不能区分真菌细胞和哺乳类动物细胞，导致严重的毒副作用。因此急需开发既有效、又安全的新型抗真菌药物。E1族大肠菌素是大肠杆菌产生的一种可形成离子通道的细菌素，它们只能杀伤大肠杆菌及亲缘关系接近的某些细菌。我们应用生物工程技术将白色念珠菌α交配信息素连接在大肠菌素Ia通道结构域上，即构成一种

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新型融合蛋白——信息菌素-CA (PMC-CA)。间接荧光标记数据显示该信息菌素可结合在真菌细胞上，实验室/田间实验数据证实信息菌素可抑制真菌生长。按分子量标化，田间实验数据证实信息菌素对稻瘟病的保护率显著高于三唑类、三环唑和稻瘟灵等现用抗稻瘟病药物的保护率。上述结果提示该融合蛋白具有发展成为农业抗真菌药物的价值。

关键词：细菌素类蛋白；交配信息素；融合抗真菌多肽
中图分类号：Q812；S435.111.4+1

Fungal pathogens represent major problems for human health and agriculture. In humans, *Candida albicans* (CA) is the most prevalent fungal pathogen, and is responsible for potentially life-threatening infections [1]. In agriculture, *Magnaporthe oryzae* causes a devastating rice blast disease that in some areas has reached epidemic levels [2–5]. About 10% to 30% of the annual rice harvest is lost due to infection by the rice blast fungus [2–5]. Besides causing huge crop losses, these fungi can produce mycotoxins that are detrimental to human and animal health [2–5].

Fungi are eukaryotic organisms and share some important features with mammalian cells [2]. Current antifungal antibiotics often can not distinguish between fungi and mammalian cells resulting in serious side effects in mammalian cells. In addition, current anti-fungal measures based on chemical control produce undesirable health and environmental consequences [2–5]. Accordingly, there is strong impetus to develop antifungal alternatives that are both safe and effective.

CA can undergo sexual mating facilitated by specific peptides, pheromones [1,2]. The α-factor pheromone induces genes in CA that are required for both mating and pathogenesis [1]. One group of pheromone-induced genes in CA encodes a set of cell surface, and secreted proteins that are required for both mating and pathogenesis [1]. Pheromones are not necessarily species specific, as previous studies have shown that α-factors of various fungi can interact with other fungal species [6]. In previous studies, we engineered a bacterial pheromone to functionally replace the ligand domain of a channel-forming bacterial toxin in order to direct bactericidal properties of the toxin toward a selected target [7–9]. In the current work, our strategy was to construct a fusion peptide with CA α-factor and colicin Ia and examine antifungal effects against certain fungal pathogens which cause devastating fungal diseases in agricultural plants. Colicin Ia is a bacterial peptide that can form ion conductive pores in cell membranes of targeted cells, causing cell swelling, and disruption [7,10–12].

1 MATERIALS AND METHODS

1.1 *Preparation of fusion molecules*

The GFRLTNFGYFEPG sequence of CA pheromone[1] were inserted into the downstream of I626 of colicin Ia using a double-stranded oligonucleotide mutagenesis (QuickChange kit, Strategene) and a Promega pSELECT-1 plasmid containing the colicin Ia gene (courtesy of P. Gosh, UCSF, USA) to form pheromonicin-CA (PMC-CA). The oligonucleotides containing the desired GFRLTNFGYFEPG mutation, were 5’-GCG AATAAG TTC TGG GGT ATT **GGG TTT CGT CTCACC AAC TTC** TAA ATA AAA TAT AAG ACAGGC-3’ and 5’-ATT GGG TTT CGT CTC ACC AACTTC **GGA TAC TTT GAA CCT GGA** TAA ATAAAA TAT AAG ACA GGC-3’ (boldface triplets represent inserted pheromone sequences). Harvested plasmid was transfected into TG1 and BL-21 *E. coli* cells to produce PMC-CA as previously described [11, 12]. The total protein concentration of elute was about 1–3 mg/mL. The specific activity of every batch protein had been tested before used in the experiment as previous studies reported [7–13]. Control derivatives were prepared similarly.

1.2 *Electrophysiological assay*

Channel-forming experiments were done on planar lipid bilayer membrane formed at 20–22 °C from asolectin (Lethicin type IIS; Sigma) as previously described [7, 10]. For preparation of a control derivative, PMC-CA (Red), the oligonucleotide used containing the desired polar amino acid substitution, was 5’-GTC TTC AGT ATTCTT **GAA** GGA **AAA** GCT TTA GGC **AAA AAA**GGG ATA GGT TTA CTG-3’ to substitute T592E, S594K, I598K and I599K residues in C8 and C9 helices of Ia channel-forming domain (boldface triplets represent inserted polar residue genes).

1.3 *In vitro growth inhibition and immunolabeling assays*

C. albicans (American Type Culture Collection, ATCC 10231), *M. oryzae* (Agriculture Culture Collection of China, ACCC 30320), Mg2005044 (South-West Agriculture University), Mg2002413 (Sichuan Agriculture

University, SAU), and field isolates collected by Division of Plant Protection, Yaan Agriculture Bureau (YAB Mg2005312, Mg2006211, Mg2008014 and Mg2008056), *Curvularia lunata* (SAU Cl2003212), *Botrytis cinerea* (SAU 2002225), *Fusarium oxysporum* (SAU Fo2005414), *Sclerotinia sclerotiorum* (SAU Sc20003175), *Fusarium solani* (SAU Fs2004116), *Fusarium moniforme* (SAU Fm2005217), *Blumeria graminis* (SAU Bg20084218), and *Aspergillus flavus* (ACCC 30302) were grown in 10 mL LB, Sabouraud liquid medium, or PDA agar medium, respectively. Cells were inoculated to an initial cell density of 1×10^6–5×10^6 cells/mL and grew at 25, 30, or 37 °C with shaking, or without shaking for 20 to 240 hrs, respectively.

Known concentrations of PMC-CA were prepared by diluting purified preparations. The dosages of amphotericin B, isoprothiolane and tricyclazole used were the maximum recommended by the manufacturers. After 20 to 70 hrs growth, the fungal pellets were collected by centrifugation for 10 min at 5,000 *g*, dried for 24 hrs at 80 °C, and the weight of dried pellets was measured [23–25].

M. oryzae infected samples growing in greenhouse and experimental fields were collected, fixed and sealed with 10% BSA/PBS, incubated with mouse anti-colicin IgG mAb (1:100), followed by FITC-labeled goat anti-mouse antibody (1:100), and observed under an optical/fluorescent microscope (Nikon 90i) with DM400, DM505 and DM565 filters.

M. oryzae cells were incubated with PMC-CA, or growth medium alone for 10 min to 600 min. Then samples were assayed by fluorescent dyes, 600 nmol/L propidium iodide and 50 nmol/L acridine orange under an optical/fluorescent microscope (Nikon 90i) with DM400, DM505 and DM565 filters.

The antifungal effect of PMC-CA was identified with morphology and ribosomal DNA-Its sequencing assays [23,24]. Spore suspensions (1×10^6 spores/mL) were spread on the slice and incubated with water (or 50 mmol/L Borate) as control, or with different volumes of PMC-CA preparation at 15 °C (for *B. graminis*) or 26 °C (for others) for 24 hrs, then 100 spores were counted and the percentage of spore germination was calculated. Mycelial plugs were incubated on PDA agar medium with 1/5 volume diluted pheromonicin preparation at 26 °C for 7 d with light, and the diameters of mycelial growth were measured to calculate the inhibition ratio: (diameter of untreated mycelial growth – diameter of pheromonnicin-treated mycelial growth)/ diameter of untreated mycelial growth × 100%. Mycelial plugs (5 mm) were incubated with Czapek medium with 1/5 volume diluted pheromonicin preparation at 26 °C for 10 d with shaking, and the cell pellets were filtered, dried for 24 hrs at 80 °C, then the weight of dried pellets were taken to calculate the mycelial growth inhibition rate: (weight of dried untreated cell pellets – weight of dried pheromonicin-treated cell pellets)/ weight of dried untreated cell pellets × 100%.

1.4 *In vivo activity*

Seeds of rice blast-sensitive and resistant cultivars (6 susceptible cultivars, "2YouMing86", "ChuanYou75535", "MingYou 6", "HongYou No.1", "YiXiang 101" and "YiXiang 3724", 3 mild-resistant cultivars, "JinYou 100", "ZhongYou 448" and "GangYou 725") were sown in $54 \times 36 \times 12$ cm^3 plastic trays (6 rows with 20 seeds/row) and grown in a greenhouse (27–30 °C day, 19–22 °C night, average humidity 80%). Fourteen days after sowing, seedlings were moved to polyethylene chambers ($0.5 \times 1.0 \times 1.0$ m^3). Leaves were sprayed with 20 mL *M. oryzae* spore suspension (10^5–10^7 spores/mL) once during plants grew 3–4 complete leaves [16–18]. Typical rice blast lesions appeared on leaves 7–8 days after infection. The plants were sprayed once with wild-type colicin Ia, an unrelated *S. aureus* pheromonicin PMC-SA, PMC-CA, and tricyclazole. The leaves were collected at 24, 72, and 120 hrs for microscopy. Ten days after spraying, the leaf blast lesions on 40 randomly selected leaves of each replicate area of all treatments and means of the four replications of each treatment were used in data analysis [16–18].

Evaluation of PMC-CA inhibition on rice blast was conducted in Yaan, Sichuan, China from June to September of 2005–2010. Yaan nursery (1,100 km^2, elevation 516–2,629 m) is located in a moist basin with average annual sun-radiation 79 kCal/cm^2, average annual rain fall 1,700–2,100 mm, average annual temperature 14–16 °C as a very typical mountain ecosystem for rice blast. In 1984–1985, the area affected by rice blast disease had reached to 106.7×10^4 hm^2 with huge harvest lost (7.5×10^8 kg) in the Sichuan area. Approximately 522 strains (ZB, 78%, ZA, 11.7%, ZC, 10.9%) of "63 family" *M. oryzae* were collected from 1987 to 1996 [25]. The test fields in the current study were scattered either in the plain, along river basin, or in mountainous terraces with an elevation of 550–900 m. All replications

(15–50 m^2) were enclosed in a randomized complete block design [16–18].

Trials of leaf blast prevention with four replications were evaluated. Cultivars with established leaf blast lesions were sprayed once with respective agents during tilling stages. Samples were collected at 24, 72, or 120 hrs for microscopy. Ten days after spraying, leaf blast lesions of 200 randomly selected leaves were measured in each replicate area of all treatments, and means of the four replications of each treatment were used in data analysis [16–18].

For trials of panicle blast prevention, replicates with established leaf blast lesions were sprayed once with water alone, tricyclazole (2 mg/mL), isoprothiolane (2 mg/mL), triadimefon (120 μg, or 1.2 mg/mL), difenoconazole (25 μg, or 250 μg/mL) or PMC-CA (10, 25, 50, or 250 μg/mL) when either 5% of plants entered into early earing period, or panicle blast appeared during grain filling period. Samples were collected at 8 or 19 d for microscopy. Sixteen, or thirty days after spraying, panicle blast lesions were measured for 200–250 randomly selected spikes, part of the spikes were collected in each replicate area of all treatments, and means of the four replications of each treatment were used in data analysis [16–18].

Lesion index and relative protection effects were derived with following equations.

Leaf lesion index = ∑[number of sick leaves (or necks) × number of scale] / [total number of investigated leaves (or necks) × 9] × 100%

Relative protection effect against leaf blast (%) = $\{1 - (CK_0 \times PT_1/CK_1 \times PT_0)\} \times 100\%$

Relative protection effect against neck blast (%) = $(CK_1 - PT_1) / CK_1 \times 100\%$

CK_0 = Lesion index of control area before treatment

CK_1 = Lesion index of control area after treatment

PT_0 = Lesion index of treated area before treatment

PT_1 = Lesion index of treated area after treatment

Collected spikes were dried at 50 °C for 24 hrs. The number of grains per spike, seed setting and kilo-grain weight of spikes collected from each replicate area of all treatments and means of the four replications of each treatment were used in data analysis [16–18].

1.5 *Statistic analysis*

All statistical analyses were manipulated with SPSS ($P < 0.05$) in accordance with the Guidelines for the field efficacy trials (1)-fungicides against leaf diseases of rice, GB/T 17980.19-2000, China Agriculture Ministry 2000.

2 RESULTS

CA α-factor pheromone precursor was introduced into a plasmid at the C-terminus of PMC-CA (Fig. 1*A*, *B*). SDS PAGE showed a band of the expected size (Fig. 1*C*) and voltage-activated channels on artificial planar lipid bilayer membranes were found with properties similar to previously described pheromonicins (upper graph, Fig. 1*D*). In contrast, a control, PMC-CA (Red), consisting of a chemically reduced derivative of PMC-CA with an altered vital structural hydrophobic element, α-helices C8-C9 (Ala580-Ile612) of colicin channel, induced only low current (lower graph, Fig. 1*D*).

Control peptides consisting of (8 μg/mL) PMC-CA (Red), PMC-CA (Rev) with reversed orientation of CA α mating pheromone (N-terminal to colicin), and wild-type colicin Ia when incubated with CA cells in liquid medium had no effect on the growth of CA cells (Fig. 1*E*). In contrast, PMC-CA at the same concentration effectively inhibited the growth of CA cells (Fig. 1*E*).

Amphotericin B treatment of CA resulted in mycelial dry weight of 60 mg. After PMC-CA treatment, the weights were 38 mg (8 μg/mL PMC-CA) and 19 mg (16 μg/mL PMC-CA), representing 36% and 68% decreases, respectively ($P < 0.01$, Fig. 1*F*). PMC-CA (Rev) control had no significant effect on the dry weight which was 56 mg (data not shown). Adding free CA pheromone to PMC-CA in increasing molar ratios from 1:1 to 100:1 blocked PMC-CA inhibition of mycelial dry weight to 33%, and 17%, respectively, in a concentration-dependent manner (Fig. 1*G*).

Incubation of CA cells with free CA α-factor pheromone alone in increasing concentrations did not inhibit CA growth (data not shown). Therefore, the competitive inhibition results support the conclusion that the binding of PMC-CA molecules to CA cells was mediated by the pheromone component of the fusion protein. Furthermore, both CA α-factor pheromone and colicin components were necessary, but neither component alone was sufficient to inhibit the growth of fungal cells.

The cytosolic contents of *M. oryzae* cells stained either by propidium iodide, a vital dye alone or by both of propidium iodide and fluorescein isothiocyanate (green) dyes indicated that control peptides, PMC-CA (Red) and PMC-CA (Rev) had no visible effects on *M.*

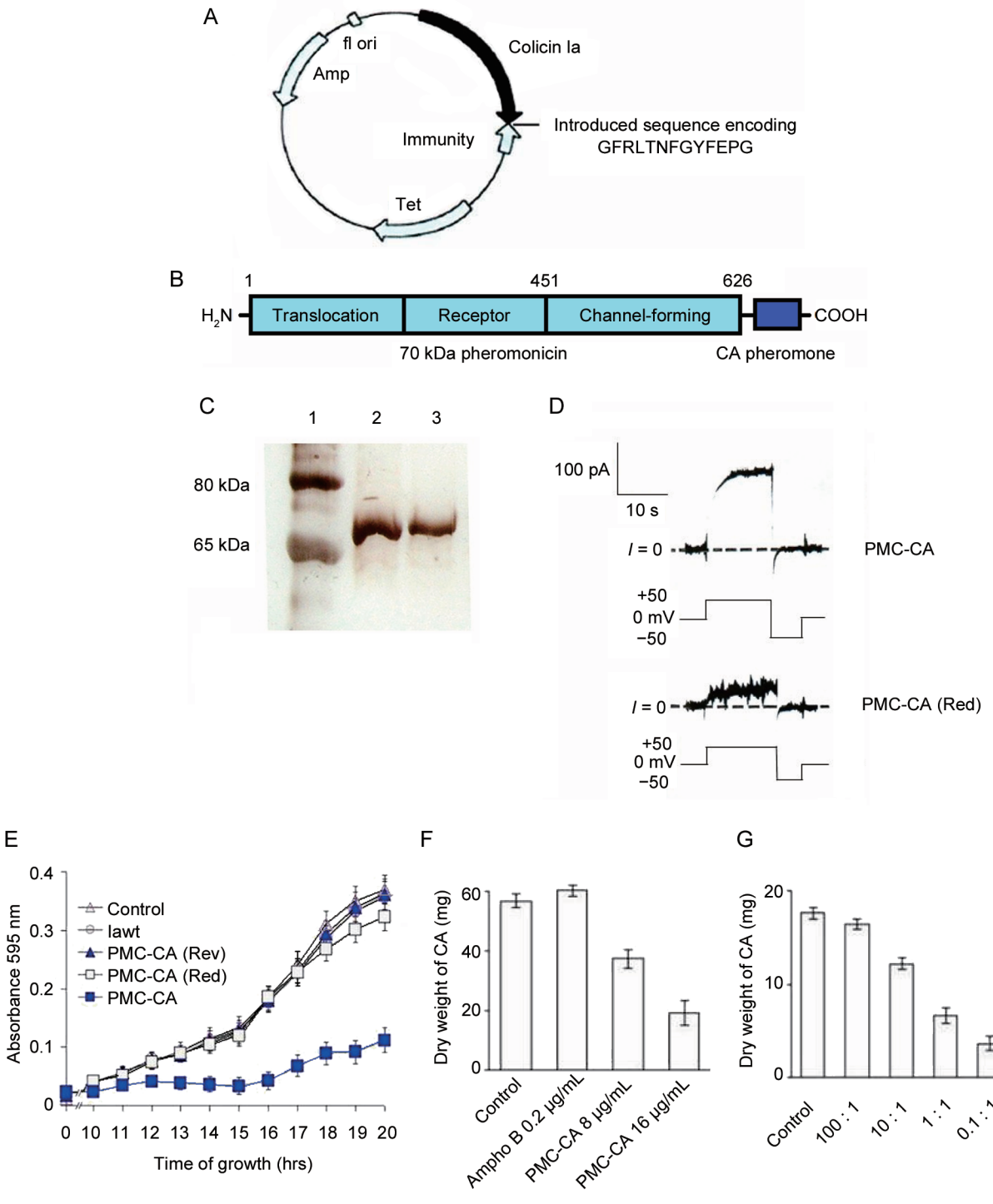

Fig. 1. Structure and basic bioactivity of PMC-CA. (*A*) 8.3 kbp colicin Ia plasmid used for site-directed mutagenesis of the colicin Ia gene, and subsequent insertion of the gene encoding the *C. albicans* α-factor pheromone. (*B*) Domain diagram of a pheromonicin construct with *C. albicans* α-factor pheromone at the C terminus. (*C*) SDS-polyacrylamide gel of PMC-CA after purification. Lane 1, molecular weight markers; lane 2, wild-type colicin Ia; lane 3, PMC-CA. (*D*) Voltage-dependent gating of PMC-CA (upper graph) and PMC-CA (Red) (lower graph) in artificial lipid bilayers. (*E*) *C. albicans* cells incubated with: control, medium alone, or 8 μg/mL each of Iawt, wild-type colicin Ia; PMC-CA (Rev), PMC-CA (Red); and PMC-CA. (*F*) Mycelial dry weight of CA treated for 72 hrs. (*G*) Concentration-dependent inhibition of PMC-CA by free CA α-factor pheromone for 30 hrs. Dry weight is plotted as a function of molar ratio from 100:1 (11 μmol/L free pheromone *vs* 0.11 μmol/L PMC-CA), to 0:1 (0 μmol/L free pheromone *vs* 0.11 μmol/L PMC-CA), respectively.

oryzae morphology (Fig. 2*A*-i). In contrast, PMC-CA resulted in clear damage with leakage of cytosolic contents, and cell disruption of *M. oryzae* cells at 20 min (Fig. 2*A*-ii, v, *B*-iii), 30–40 min (Fig. 2*A*-iii, vi, *B*-iv), and 60 min (Fig. 2*A*-iv, vii, *B*-v). Inside the same *M. oryzae* cell, the infiltration of vital dye aggravated as a time function of incubation with PMC-CA in 60 min (Fig. 2*A*-ii to iv). The time course of mycelial cells presented more profound morphological evidence to verify above identification. Since incubated with PMC-CA less than 10 min (Fig. 2*B*-ii), the FITC-stained cells (green) were eventually replaced by PI-stained cells (yellow-orange-red) with deformed shapes, swelling sizes, cell membrane perforation and leaked cytosol contents within 60 min (Fig. 2*B*-v). The data supported the conclusion that PMC-CA permanently inhibited growth of *M. oryzae* cells, and this effect was associated with an increase in permeability to a vital dye.

The inhibitory activity of PMC-CA was measured against 9 pathogenic imperfecti fungi which caused devastating diseases of crops, vegetables and fruits, such as rice blast, maize curvularia leaf spot, rape sclerotinia, and tomato grey mould etc. The results of spore germination and mycelial growth inhibition assays indicated that in the presence of PMC-CA, about 50%–100% of spore germination and mycelial growth of *M. oryzae*, *C. lunata*, *B. cinerea*, *F. oxysporum*, *S. sclerotiorum*, *F. solani, F. moniforme*, *B. graminis* and *A. flavus* were inhibited (Fig. 3).

The microscopic observations were reflected in cell growth of *M. oryzae* as shown in Fig. 4. Free colicin Ia and isoprothiolane did not significantly alter the cell growth. In contrast, PMC-CA significantly inhibited the cell growth by 65% (Fig. 4).

To evaluate the agricultural potential of PMC-CA, *M. oryzae* cells were sprayed onto *M. oryzae*-susceptive and mild-resistant rice cultivars under greenhouse and experimental field conditions. After infection, rice plants were sprayed with water alone, wild-type colicin Ia control or PMC-CA. Treated leaves were collected at 24, 72, and 120 hrs later. Leaves were sectioned and mounted on the slices for hematoxylin/eosin staining (H&E) or indirect immunolabeling staining (IIS). A typical leaf from a *M. oryzae*-infected cultivar with invasive fungus is shown in Fig. 4*B* [15]. Untreated, infected control leaves, stained with mouse anti-colicin antibody and fluorescent second antibody (Fig. 4*C*) or infected leaves, with PMC-CA treatment, without anti-colicin, but with second fluorescent antibody (Fig. 4*D*) showed hyphae and plant cells by blue autofluorescence, but no green antibody fluorescence indicating that the antibodies did not bind non-specifically to rice or fungal antigens. In contrast, Fig. 4*E* shows that after PMC-CA treatment followed by first and fluorescent second antibody exposure, hyphae developed green fluorescence.

Figure 4*F* shows an untreated, infected rice neck with bulbous invasive hyphae stained by H&E. Similar invasive hyphae was not stained by anti-colicin antibody (Fig. 4*G*). In contrast, 8 days after treatment with PMC-CA followed by anti-colicin and fluorescent second antibody staining, hyphae still developed green fluorescence (Fig. 4*H*). Nineteen days after PMC-CA treatment, invasive hyphae appeared deformed, and shrunken by H&E staining (Fig. 4*I*).

After the rice blast lesions appeared on leaves in the fields, the rice plants were sprayed once with field water alone, or field water plus (10 μg/mL) of PMC-CA,

Table 1. Damage index of treatments against leaf blast (MingYou 6, susceptible cultivar)

Investigation time	Number of blast leaves							Incidence rate	Damage index	Protective efficiency
	Class	0	1	3	5	7	9			
1 days before spray		152	27	14	6	1		24%	5.88	
14 days after spray	**Control**	89	57	90	9	7	8	55.5%	17.36	
	Tricyclazole 2 mg/mL	172	10	12	8			14%	4.22	75.83%
	PMC-CA 10 μg/mL	167	12	13	8			16.5%	5.05	70.94%

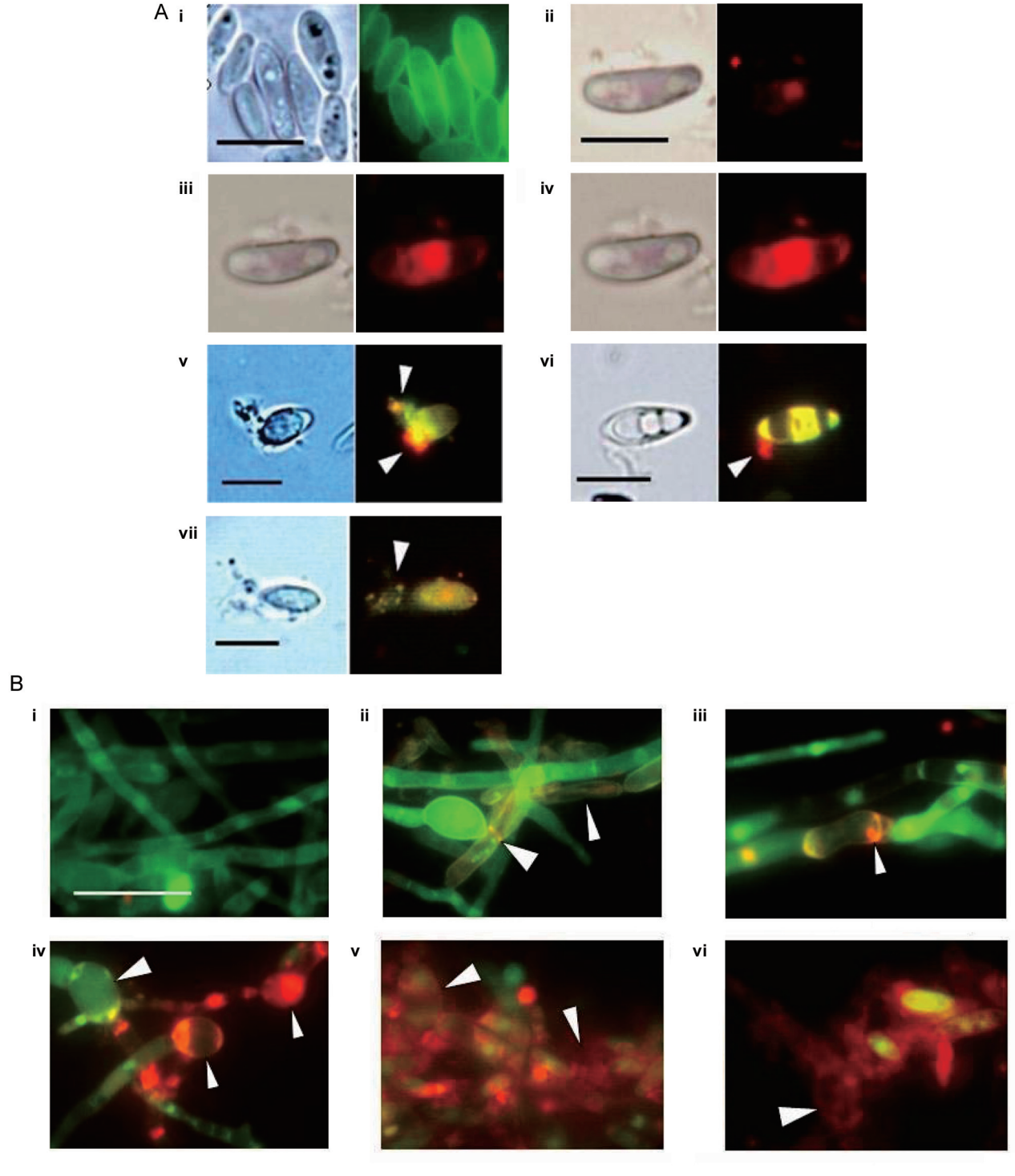

Fig. 2. Cellular damage of plant fungal pathogens induced by PMC-CA. Photomicrographs of untreated *M. oryzae* cells at 120 min (*A*-i, *B*-i). PMC-CA treatment at (*A*-ii and *B*-ii) 10 min, (*A*-iii, *A*-v and *B*-iii) 20 min, (*B*-iv) 30 min, (*A*-iii and *A*-vi) 40 min, (*A*-iv, *A*-vii and *B*-v) 60 min and (*B*-vi) 120 min. Left panels, optical images; right panels, fluorescent images obtained with either PI alone (*A*-ii to *A*-iv) or both of PI and FITC (*A*-i, *A*-v to *A*-vii and *B*) applied. White arrows, membrane impairement, or leakage of cytosolic contents. (*A*-ii to *A*-iv) 20–60 min sequential images of a fungal cell incubated with PMC-CA and stained by PI alone. Scale bars, 10 μm (*A*-i, *A*-v to *A*-vii), 5 μm (*A*-ii), 20 μm (*B*-i).

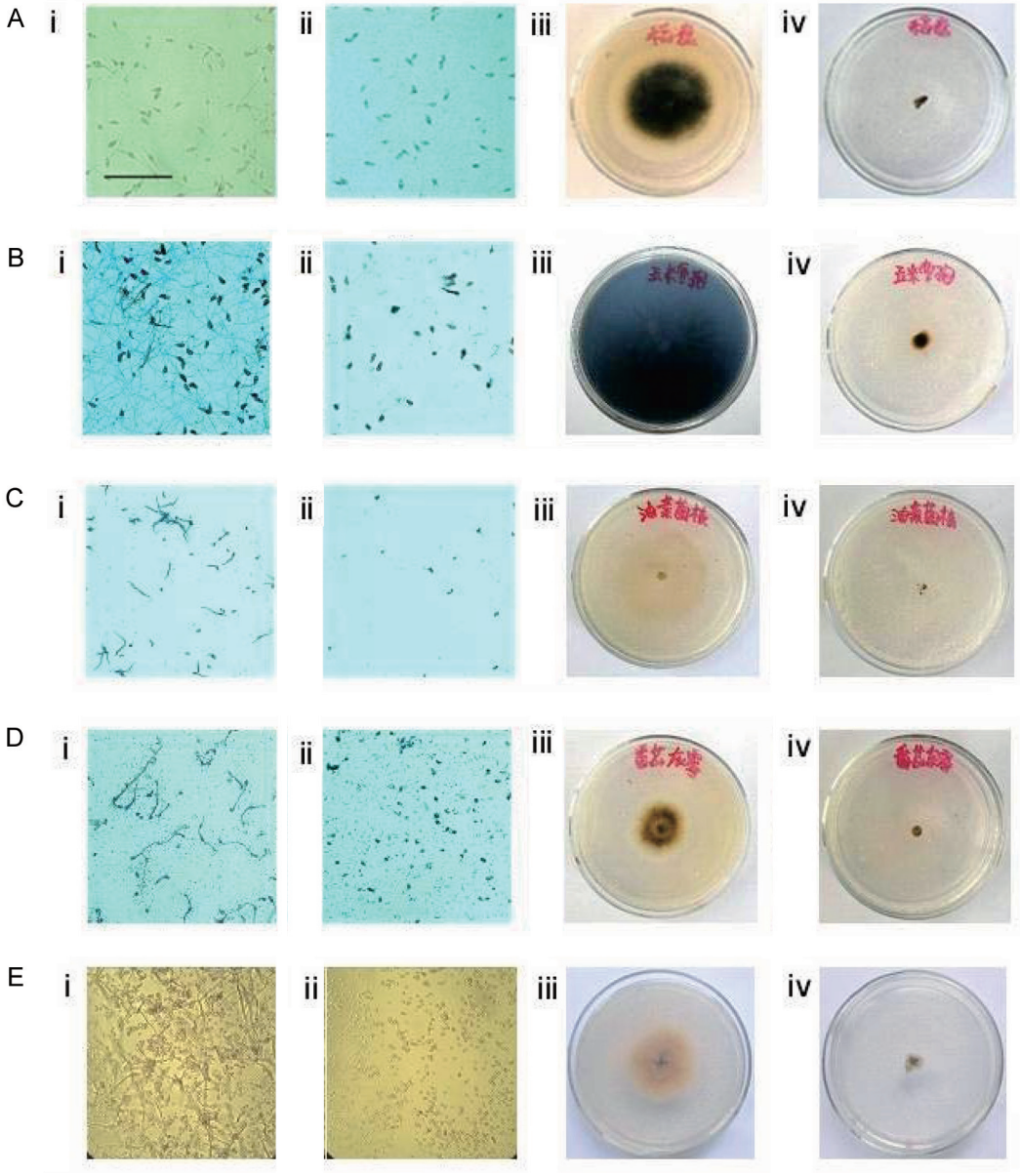

Fig. 3. Spore germination and mycelical growth of plant fungal pathogens were inhibited by PMC-CA. Spore germination and mycelial growth of (*A*) *Magnaporthy oryzae*, (*B*) *Curvular lunata*, (*C*) *Sclerotinia sclerotiorum*, (*D*) *Botrytis cinerea* and (*E*) *Asperigillus flavus* were treated by medium alone (i and iii), or PMC-CA (ii and iv). Scale bar in *A*-i, 50 μm.

or tricyclazole (2 mg/mL). Ten days after spraying, leaf blast lesions were identified on 200 randomly selected leaves of each replicate of all treatments and means of the four replications of each treatment were used in data analysis [16–18]. Table 1 shows that PMC-CA shared about the same protective effects as that of tricyclazole against leaf blast disease in the fields.

During 5% of rice plants with established leaf blast lesions entered into the earing period, rice plants were sprayed once with field water alone, or field water with PMC-CA (10, 25, 50 and 250 μg/mL), or tricyclazole (2 mg/mL), or isoprothiolane (2 mg/mL), or triadimefon (120 μg, and 1.2 mg/mL), or difenoconazole (25 and 250 μg/mL) in experimental fields. 30 days after spraying, panicle blast lesions were identified on 200–250 randomly selected spikes. PMC-CA exposure to panicle blast resulted in either similar protective effects as that of tricyclazole, or isoprothiolane; or significant protective effects than that of tricyclazole, or triadimefon, or difenoconazole treatments in different seasons (Table

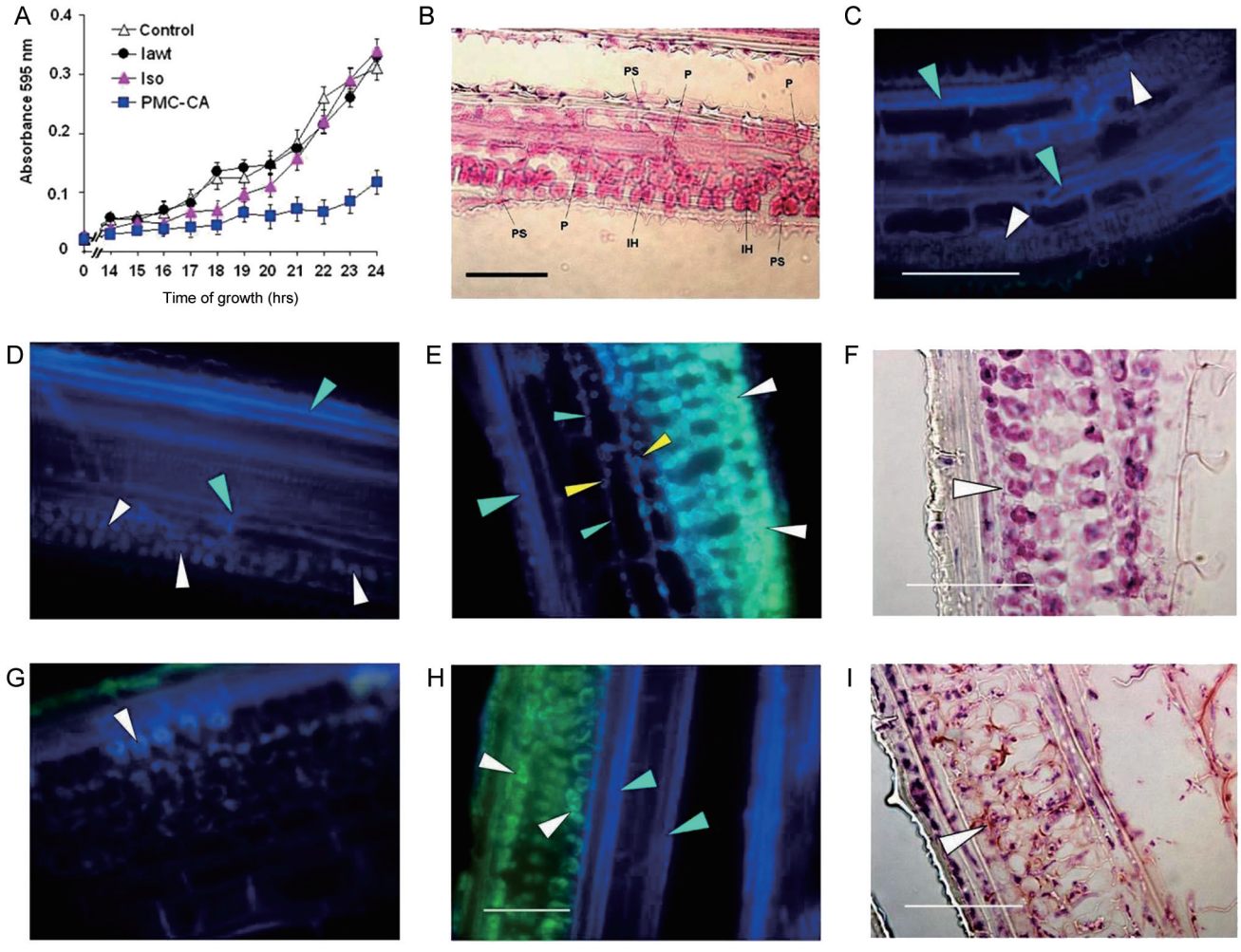

Fig. 4. Interaction between PMC-CA molecules and *M. oryzae* cells in infected plant tissues. (*A*) Growth of *M. oryzae* cells incubated with Control, medium alone; Iso, Isoprothiolane (50 μg/mL); and Iawt, wild-type colicin Ia (25 μg/mL); and PMC-CA (25 μg/mL). (*B*) Photomicrographs of rice leaf. (P) primary *M. oryzae* hyphae, (PS) differentiated bulbous hyphae, (IH) invasive hyphae by H&E staining. (*C*–*G*) Rice leaf cells and invasive *M. oryzae* hyphae 1 day after treatment. (*C*) Control, medium without PMC-CA treatment followed by mouse anti-colicin antibody against PMC-CA, and FITC-labeled goat anti-mouse antibody (green). *M. oryzae* invasive hyphae (white arrows), (*D*) with PMC-CA treatment, without anti-colicin, but with second fluorecent antibody. Green arrows, auto-fluorescence of normal plant cells. (*E*) PMC-CA treatment followed by anti-colicin and fluorescent second antibody. Invasive hyphae (white arrows), primary invasive hyphae (yellow arrows). (*F*–*H*) Rice necks 8 days after treatment. (*F*) Untreated, infected rice neck stained by H&E, (*G*) without PMC-CA treatment, but with anti-colicin and fluorescent second antibody, (*H*) treated with PMC-CA followed by anti-colicin and fluorescent second antibody. Hyphae (white arrows), adjacent normal rice plant cells (green arrows). (*I*) Infected neck, 19 days after PMC-CA treatment stained with H&E. Invasive hyphae (white arrows). Scale bars in *B*, *C*, *F*, *H*, and *I*, 50 μm.

2*A*-*B*, 3, 4, 5). Kilo-grain weight data from PMC-CA field trials were significantly, 135%–150%, higher than untreated controls and 21%–47% higher than tested fungicide treatments (Fig. 5 and Table 6).

Until panicle blast lesions established in early grain filling period, rice plants were sprayed once with field water alone, or field water with PMC-CA (25, and 250 μg/mL), or triadimefon (1.2 mg/mL) in experimental fields. 16 days after spraying, panicle blast lesions were identified on 200 randomly selected spikes. PMC-CA exposure to panicle blast resulted in significant protective effects than that of triadimefon treatment (Table 2,

Table 2. Damage index of treatments against panicle blast

A (YiXian 101, susceptible cultivar)

Investigation time	Number of blast leaves							Incidence rate	Damage index	Protective efficiency
	Class	0	1	3	5	7	9			
30 days after spray	**Control**	53	84	60	29	18	6	79%	26.2	
	Tricyclazole 2 mg/mL	106	84	30	15	13	3	58%	16.3	38%
	Isoprothiolane 2 mg/mL	115	80	27	14	12	3	55%	15.2	42%
	PMC-CA 10 μg/mL	93	92	37	18	11	1	63%	16.9	36%
	PMC-CA 25 μg/mL	101	106	27	13	4	0	60%	12.5	53%
	PMC-CA 50 μg/mL	108	89	29	22	9	0	58%	15.5	41%

B (YiXian 3724, susceptible cultivar)

Investigation time	Number of blast leaves							Incidence rate	Damage index	Protective efficiency
	Class	0	1	3	5	7	9			
30 days after spray	**Control**	85	72	41	15	12	1	62%	17.85	
	Tricyclazole 2 mg/mL	115	86	15	8	5	0	50%	9.99	44%
	Triadimefon 1.2 mg/mL	104	92	21	4	2	0	53%	9.41	47%
	Triadimefon 120 μg/mL	91	61	24	11	5	0	53%	14.0	22%
	Difenoconazole 250 μg/mL	100	75	27	10	2	0	53%	11.94	33%
	Difenoconazole 25 μg/mL	89	79	42	18	4	0	62%	15.47	13%
	PMC-CA 25 μg/mL	149	34	17	4	0	0	27%	5.72	68%
	PMC-CA 250 μg/mL	183	26	9	4	0	0	18%	3.65	80%

C (YiXian 3724, susceptible cultivar)

Investigation time	Number of blast leaves							Incidence rate	Damage index	Protective efficiency
	Class	0	1	3	5	7	9			
16 days after spray	**Control**	51	120	38	6	3	2	77%	15.30	
	Triadimefon 1.2 mg/mL	103	75	45	14	0	0	57%	13.13	14%
	PMC-CA 25 μg/mL	158	44	6	0	0	0	24%	3.31	78%
	PMC-CA 250 μg/mL	163	36	2	0	0	0	19%	2.32	85%

Table 3. Damage index of treatments against panicle blast (YiXian 101, susceptible cultivar)

1 replicate			2 replicate		
Code	**Invest. number**	**Damage index**	**Code**	**Invest. number**	**Damage index**
A	250	26.2	**B**	250	19.37
D	250	16.88	**A**	250	27.58
B	250	16.28	**F**	250	16.72
C	250	15.22	**E**	250	18.93
F	250	15.5	**D**	250	20.01
E	250	12.48	**C**	250	14.85
3 replicate			**4 replicate**		
Code	**Invest. number**	**Damage index**	**Code**	**Invest. number**	**Damage index**
E	250	8.64	**F**	250	17.5
D	250	18.91	**E**	250	12.99
A	250	26.29	**D**	250	21.13
C	250	20.05	**C**	250	20.21
B	250	21.25	**B**	250	14.08
F	250	18.48	**A**	250	45.23

A, Control; B, Tricyclazole (2 mg/mL); C, Isoprothiolane (2 mg/mL); D, PMC-CA (10 μg/mL); E, PMC-CA (25 μg/mL); F, PMC-CA (50 μg/mL). There are significant differences between control and each treatment ($P < 0.05$). There are no significant differences among 5 treatments.

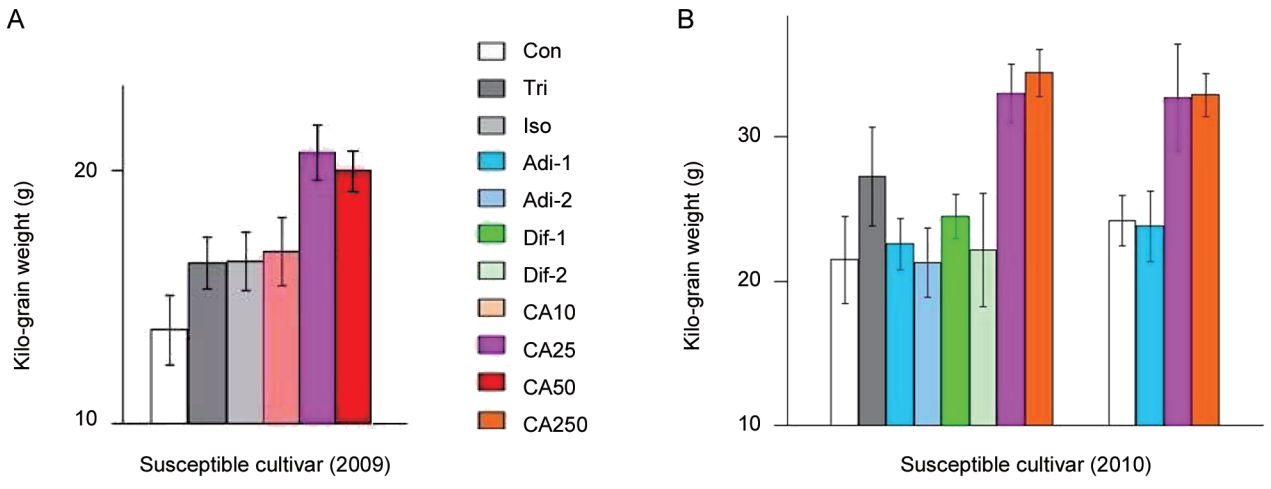

Fig. 5. Protective efficiency of PMC-CA against fungal infection in the field test. Kilo-grain weight of panicle blast in field-grown rice treated with (*A*) Con, water control; Tri, tricyclazole (2 mg/mL); Iso, isoprothiolane (2 mg/mL); CA10, CA25 and CA50 (10, 25 and 50 μg/mL) (Yi Xiang 101, susceptive cultivar) and (*B*) (left part) Con, water control; Tri, tricyclazole (2 mg/mL); Adi-1, triadimefon (1.2 mg/mL), Adi-2, triadimefon (120 μg/mL), Dif-1, defenoconazole (250 μg/mL), Dif-2, defenoconazole (25 μg/mL), CA25 and CA250 (25 and 250 μg/mL)(Yi Xiang 3724, susceptive cultivar), thirty days before sample collection, or (right part) Con, water control; Adi-1, triadimefon (1.2 mg/mL), CA25 and CA250 (25 and 250 μg/mL)(Yi Xiang 3724, susceptive cultivar), sixteen days before sample collection.

Table 4. Damage index of treatments against panicle blast (YiXian 3724, susceptible cultivar)

1 replicate			2 replicate		
Code	**Invest. number**	**Damage index**	**Code**	**Invest. number**	**Damage index**
A	200	12.19	**B**	200	6.74
D	200	9.99	**A**	200	17.85
B	200	5.67	**F**	200	11.06
C	200	5.72	**E**	200	11.94
F	200	10.11	**D**	200	10.17
E	200	12.32	**C**	200	4.32
G	200	11.52	**G**	200	9.41
H	200	14.00	**H**	200	11.81
3 replicate			**4 replicate**		
Code	**Invest. number**	**Damage index**	**Code**	**Invest. number**	**Damage index**
E	200	8.78	**F**	200	14.29
D	200	7.57	**E**	200	9.3
A	200	10.26	**D**	200	7.94
C	200	7.57	**C**	200	7.94
B	200	5.23	**B**	200	3.65
F	200	15.47	**A**	200	9.39
G	200	7.27	**G**	200	7.25
H	200	11.55	**H**	200	9.24

A, Control; B, Tricyclazole (2 mg/mL); C, Triadimefon (1.2 mg/mL); D, Triadimefon (120 μg/mL); E, Difenoconazole (250 μg/mL); F, Difenoconazole (25 μg/mL); G, PMC-CA (25 μg/mL); H, PMC-CA (250 μg/mL). There are no significant differences between Triadimefon (120 μg/mL)/Difenoconazole (25 μg/mL and 250 μg/mL) treatments and control. There are significant differences between Triadimefon (1.2 mg/mL)/PMC-CA treatments and control ($P < 0.05$). There are no significant differences between PMC-CA treatments and Triadimefon (1.2 mg/mL) treatment. There are significant differences among PMC-CA treatments and the rest treatments ($P < 0.05$).

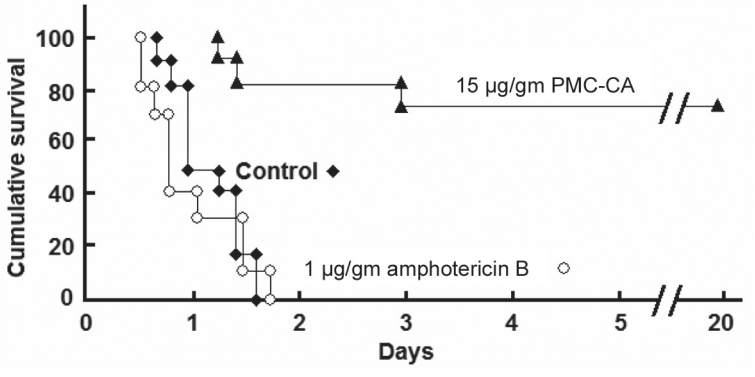

Fig. 6. *In vivo* activity of PMC-CA against fatal fungal infection. Cumulative survival of mice after *C. albicans* infection. One hour after intraperitoneal injection of *C. albicans*, mice were injected intraperitonealy with amphotericin B at 1 μg/gm or PMC-CA at 15 μg/gm once a day, in separate groups.

Table 5. Damage index of treatments against panicle blast (YiXian 3724, susceptible cultivar)

1 replicate			2 replicate		
Code	**Invest. number**	**Damage index**	**Code**	**Invest. number**	**Damage index**
A	200	15.30	**B**	200	4.35
D	200	13.13	**A**	200	15.03
B	200	3.31	**D**	200	10.8
C	200	2.22	**C**	200	2.32
3 replicate			**4 replicate**		
Code	**Invest. number**	**Damage index**	**Code**	**Invest. number**	**Damage index**
D	200	9.45	**D**	200	6.15
A	200	10.54	**C**	200	2.29
C	200	2.66	**B**	200	3.11
B	200	1.54	**A**	200	10.45

A, Control; B, PMC-CA (25 μg/mL); C, PMC-CA (250 μg/mL); D, Triadimefon (1.2 mg/mL). There are significant differences between control and each treatment ($P < 0.05$). There are significant differences between PMC-CAs and triadimefon treatments ($P < 0.05$).

Table 6. Kilo-grain weight of treatments against panicle blast (YiXian 3724, susceptible cultivar)

	Replicate			
A	22.5	19.79	25.24	18.37
B	32.74	37.15	32.98	34.86
C	31.31	32.11	33.44	35.12
D	29.86	28.6	22.26	28.38
E	22.12	24.78	20.49	22.96
F	24.02	19.36	22.6	19.12
G	22.47	24.21	25.78	25.49
H	21.66	21.57	17.98	27.43

Report

VAR00002

VAR00001	Mean	N	Std. deviation
A	21.4750	4	3.03897
H	34.4325	4	2.04466
G	32.9950	4	1.66694
B	27.2750	4	3.40632
C	22.5875	4	1.78549
D	21.2750	4	2.42225
E	24.4875	4	1.50808
F	22.1600	4	3.90911
Total	25.8359	32	5.49173

A, Control; B, Tricyclazole (2 mg/mL); C, Triadimefon (1.2 mg/mL); D, Triadimefon (120 μg/mL); E, Difenoconazole (250 μg/mL); F, Difenoconazole (25 μg/mL); G, PMC-CA (25 μg/mL), H, PMC-CA (250 μg/mL). There are no significant differences between Triadimefon (120 μg/mL)/Difenoconazole (25 μg/mL and 250 μg/mL) treatments and control. There are significant differences between above treatments/control and Triadimefon (1.2 mg/mL)/Tricyclazole (2 mg/mL) treatments ($P < 0.05$). There are significant differences among PMC-CA treatments and the rest treatments/control ($P < 0.05$).

Table 7. Kilo-grain weight of treatments against panicle blast (YiXian 3724, susceptible cultivar)

	Replicate			
A	22.53	25.94	22.94	25.46
B	34.08	35.96	34.08	27.51
C	32.18	32.7	31.22	34.73
D	25.39	24.92	20.21	24.69

Report

VAR00002

VAR00001	Mean	N	Std. deviation
A	24.2175	4	1.731134
B	32.9075	4	3.705864
C	32.7075	4	1.481157
D	23.8025	4	2.412652
Total	28.40875	16	5.061247

A, Control; B, PMC-CA (250 μg/mL); C, PMC-CA (25 μg/mL); D, Triadimefon (1.2 mg/mL). There is no significant difference between Triadimefon (1.2 mg/mL) and control. There are significant differences between PMC-CA treatments and Triadimefon treatment/control ($P < 0.05$).

5). Kilo-grain weight data from PMC-CA field trials were significantly, 135%, higher than untreated controls and 137% higher than triadimefon treatment (Fig. 5*B*, Table 7).

The results of the present study support the conclusion that the antifungal activity of PMC-CA (70 kDa) against rice blast disease was at least 10,000–20,000 times greater, on a molar basis, than that of tricyclazole (0.19 kDa), tested azoles (0.29–0.4 kDa), or isoprothiolane (0.29 kDa) in field studies.

Our *in vitro* and *in vivo* results demonstrate that the antifungal activity of PMC-CA was up to 10,000-fold greater, on a molar basis, than that of the currently available antifungal agents, amphotericin B, isoprothiolane, tricyclazole or azole derivatives against the strains of *C. albicans*, and *M. oryzae*. All these data indicate that PMC-CA is potent and effective in both the laboratory setting and under actual field conditions.

To prevent the occurrence of panicle blast disease, azoles or isoprothiolane have to be used at the earing period (at least 30 days before the appearance of panicle blast lesions), otherwise the panicle blast outbreak would be very hard to prevent, if above agents used later than that critical time [18]. In contrast, our damage index and kilo-grain weight data indicated that pheromonicin is capable to alleviate such severe impairments, even being sprayed as late as panicle blast have appeared in the fields (Table 2*C*, 5, 7). Also our morphological findings indicated that in the plant tissues, invasive hyphae have been destroyed with pheromonicin sprayed on the surface of plants (Fig. 4*F–I*). Our findings suggest that pheromonicin is not only to prevent the occurrence of panicle blast, as well as what routine fungicides behaved, but also probably be able to alleviate emerged panicle impairments via killing invasive hyphae in the plants.

3 DISCUSSION

Previous studies found that properties of colicin are, (1) the colicin from intestinal bacteria can diffuse through the bowel wall into the blood stream and can contribute to the heat-resistant bactericidal property of normal blood [19]; (2) it is a non-haemolytic protein and has no endotoxin toxicity [20]; (3) it can be inactivated by slices of kidney and liver and destroyed by proteolytic enzymes, such as trypsin and pepsin [20, 21]. The results of oral-intake rodent acute/subacute toxicity and genotoxicity tests (Toxicity Analysis Center, School of Public Health, Sichuan University, report SP200900101) demonstrated that pheromonicin is a nontoxic substance (data not shown). In mice with inoculated disseminated

candidiasis, all mice in the spared stock solution alone and amphotericin B (1 μg/gm/day) groups died in less than 2 d. In contrast, 70% of the mice with intraperitoneal PMC-CA (15 μg/gm/day) survived the 20-day experimental period and there was no microscopic evidence of severe necrosis or inflammation in vital organs of survived mice (Fig. 6). These findings suggest that with adaptation from wild-type colicin, pheromonicin almost behaves not only as a nontoxic protein to mammalian hosts, despite it was taken through either intestines or circulation, but also as a biosafty agent to environment due to it would be degraded by proteolytic enzymes [8, 9, 13, 19–21].

Previous studies found that coilicin Ia seemed to behave as a "saturated" activity in the lipid bilayer membrane and against bacterial infections, that is, the activities of higher dosages (over hundred μg/mL) were not so effective than that of lower dosages (20–50 μg/mL) [7–9]. We thought it is due to limited activating sites on the cell membranes and further studies would be performed to explore this phenomenon in details.

The fertile strains of both mating types of *M. oryzae* have been found in the Indian Himalayas region, the origin of rice, and rice blast. However, the sexual stage has not been confirmed yet in the field [4, 6, 22]. The indirect immunolabeling assay data indicated that PMC-CA bound to membranes of not only laboratory grown but also greenhouse or field-grown fungal cells as well as laboratory-cultured or wild-type *M. oryzae* infected rice leaves and neck parts. The fact that PMC-CA interacted with *M. oryzae* regardless of the origin and growth conditions of the fungus, indicates that the observed binding of PMC-CA to *M. oryzae* cells was not an artifact of any particular laboratory or field conditions.

* * *

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