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A novel antimicrobial protein for plant protection consisting of a *Xanthomonas oryzae* harpin and active domains of cecropin A and melittin

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Summary

Discoveries about antimicrobial peptides and plant defence activators have made possible the *de novo* and rational design of novel peptides for use in crop protection. Here we report a novel chimeric protein, Hcm1, which was made by linking the active domains of cecropin A and melittin to the hypersensitive response (HR)-elicitor Hpa1 of *Xanthomonas oryzae* pv. *oryzicola*, the causal agent of rice bacterial leaf streak. The resulting chimeric protein maintained not only the HR-inducing property of the harpin, but also the antimicrobial activity of the cecropin A-melittin hybrid. Hcm1 was purified from engineered *Escherichia coli* and evaluated in terms of the minimal inhibitory concentration (MIC) and the 50% effective dose (ED_{50}) against important plant pathogenic bacteria and fungi. Importantly, the protein acted as a potential pesticide by inducing disease resistance for viral, bacterial and fungal pathogens. This designed drug can be considered as a lead compound for use in plant protection, either for the development of new broad-spectrum pesticides or for expression in transgenic plants.

Introduction

The human food supply depends on agricultural crop production, which can be severely reduced by plant diseases caused by fungal, bacterial, viral and nematode pathogens (Alfano and Collmer, 1996; Ferre *et al.*, 2006; Marcos *et al.*, 2008). Synthetic chemical pesticides continue to play a prominent role in attempts to protect plants from disease and thus maintain crop productivity (Knight *et al.*, 1997; Marcos *et al.*, 2008). However, many are toxic and/or carcinogenic to humans and other animals, and some cause serious, long-term environmental pollution. In addition, their efficacy can be lost upon the emergence of chemical-resistant pathogens (Knight *et al.*, 1997; Makovitzki *et al.*, 2007; Marcos *et al.*, 2008). Current day demands for food and environmental safety as well as food security require a novel pesticide that shows high antimicrobial activity, yet is safe, non-toxic and non-polluting, to replace the traditional synthetic chemical pesticide in crop protection.

Recently, antimicrobial peptides (AMPs) have been received increased attention (Ali and Reddy, 2000; Marcos *et al.*, 2008; Melo *et al.*, 2009). AMPs are found in variety of species, including insects, plants and animals (Habermann, 1972; Andreu *et al.*, 1983; Lehrer *et al.*, 1993); cecropin A and melittin are two that have been characterized. Cecropin A, isolated from the haemolymph of the cecropia moth, is a component of the immune response in insects that shows broad spectrum activity against bacteria, fungi, enveloped viruses, and tumour cells (Andreu *et al.*, 1983; Cavallarin *et al.*, 1998; Hancock, 2001; Shai, 2002). Its mechanism of action relies on the α-helix (WKLFKKILKVL) at the C-terminus, a highly conserved 11-residue sequence that targets the bacterial membrane and disturbs bilayer integrity either by disruption or by pore formation (Andreu *et al.*, 1983; Hancock, 2001; Shai, 2002; Ferre *et al.*, 2006; Makovitzki *et al.*, 2007). Melittin is a 26-residue linear peptide isolated from bee venom; it contains the characteristic structure of membrane-bound cytolytic and *trans*-membrane helices, with a hydrophobic N-terminus and a hydrophilic C-terminus (Hristova *et al.*, 2001; Allende *et al.*, 2005; Glättli *et al.*, 2006; Raghuraman and Chattopadhyay, 2007). An α-helix in melittin, AVLKVLTTGL, has been shown to be the active domain against bacterial and human red blood cells (Hristova *et al.*, 2001; Ferre *et al.*, 2006; Glättli *et al.*, 2006; Raghuraman and Chattopadhyay, 2007). A hybrid peptide created by joining the α-helix structures of the two peptides, cecropin A and melittin, shows a better antimicrobial spectrum than

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cecropin A, and less haemolytic activity than melittin (Cavallarin *et al*., 1998; Ali and Reddy, 2000; Ferre *et al*., 2006). However, the high production cost of such long peptides and their sensitivity to protease degradation have limited their attractiveness as pesticides in plant protection (Marcos *et al*., 2008). In addition, few AMPs have been shown to activate innate plant immunity for plant protection.

Plant immunity is now conceptualized in terms of two defence layers. The first, called PTI (PAMP-triggered immunity), relies on the perception of pathogen- (or microbe-) associated molecular patterns (PAMPs or MAMPs) via pattern recognition receptors (PRRs) at the plant cell's surface. The second is ETI (effector-triggered immunity), in which plants use additional, intracellular receptors (such as *R*-gene products) to perceive effectors secreted by the pathogen and/or the effects of these effectors on suppressing the plant's PTI (Jones and Dangl, 2006). Compounds designed for use in plant protection against pathogen infection are likely to be most effective if they activate innate plant immunity as well as possess antimicrobial activity (Molina *et al*., 1998). One component of plant immunity is the hypersensitive response (HR), a rapid, local defence-related programmed cell death (Dong *et al*., 1999; Heath, 2000) that is triggered by effectors that are produced by microbial pathogens and recognized by the plant. Therefore, HR-elicitors are candidates for use in plant protection. One of the first identified HR-elicitors is HrpN, a Gly-rich, Cys-lacking and heat-stable harpin produced by the apple and pear fire blight pathogen *Erwinia amylovora* (Wei *et al*., 1992). Different harpins have been found in Gram-negative plant pathogenic *Erwinia* (Wei *et al*., 1992), *Pseudomonas* (He *et al*., 1993), *Xanthomonas* (Zou *et al*., 2006) and *Ralstonia solanacearum* (Arlat *et al*., 1994).

When the HR is elicited by harpins, multiple signalling pathways are activated, including those of salicylic acid (SA), jasmonic acid (JA), ethylene and abscisic acid (ABA) (Clarke *et al*., 2005). In addition, ion fluxes (Groover and Jones, 1999), callose disposition (Desikan *et al*., 1998) and the generation of reactive oxygen species (ROSs) (Doke, 1983) accompany increased HR marker gene expression, e.g. *HIN1*, *HSR203J* and *PR1-a* (Gopalan *et al*., 1996; Bowling *et al*., 1997; Pontier *et al*., 1998; Takahashi *et al*., 2004). Plants treated with harpins at an early growth stage show systemic acquired resistance (SAR) against pathogens and insects, and exhibit benefits in both growth and yield (Dong *et al*., 1999; Chen *et al*., 2008). In addition, it has been shown that activating the SAR in combination with fungicide application can result in a synergistic effect in protecting plants against pathogens (Molina *et al*., 1998). The harpins currently used as plant defence activators have no antimicrobial properties (Zhao *et al*., 2006). However, adding an antimicrobial activity synthetically could create a molecule that would be more effective in controlling plant disease through the type of synergism mentioned above.

Harpins are secreted from bacteria via a type-III secretion system (T3SS) into plant cells (Alfano and Collmer, 1996). They have been shown to bind to bilayer membranes (Lee *et al*., 2001; Racapé *et al*., 2005), as do cecropin A and melittin mentioned above, and part of the harpin structure is recognized as an HR-inducing PAMP by an as yet unknown plant defence-related receptor (Engelhardt *et al*., 2009; Haapalainen *et al*., 2011). Indeed, the α-helices at the C-terminus of HpaG from *X. axonopodis* pv. *glycines*, the soybean bacterial blight pathogen, and Hpa1, its homologue in rice bacterial leaf streak pathogen *X. oryzae* pv. *oryzicola*, are essential for HR induction in tobacco (Oh *et al*., 2007; Ji *et al*., 2010). This led us to propose that a chimeric protein consisting of Hpa1 plus the active domains of cecropin A and melittin might show both the HR induction typical of harpins, as well as the antimicrobial activity demonstrated for cecropin A and melittin.

Towards this objective, we here provide evidence that a novel chimeric protein, Hcm1, consisting of Hpa1 joined to the active domains of cecropin A and melittin, elicited the HR in tobacco and inhibited *in vitro* not only the growth of Gram-negative and Gram-positive bacteria, but also the germination of spores of plant pathogenic fungi. A polylinker between Hpa1 and cecropin A, and a flexible hinge between cecropin A and melittin, were essential for the dual function of the fused protein. Application of this protein activated SAR in plants and reduced disease severity caused by representative bacterial, fungal and viral pathogens, suggesting that Hcm1 is a novel molecule for use in developing new pesticides that show the synergism of both antimicrobial activity and HR activation in a single molecule. In addition, the gene encoding Hcm1 could be expressed in transgenic plants, possibly under a pathogen-induced promoter rather than a constitutive one, to develop new cultivars with increased resistance to plant diseases.

Results

Rational design for antimicrobial proteins

The HR induction in tobacco by $Hpa1_{Xoo}$ from *X. oryzae* pv. *oryzae*, the causal agent of rice bacterial blight, is attributed to two α-helices involved in coiled-coil protein interactions, SEKQLDQLLCQLISALLQ and PFTQMLM HIVGEILQAQ, at the N- and C-termini of the protein respectively (Ji *et al*., 2010). The use of SMART software (http://smart.embl-heidelberg.de) to analyse Hpa1 of *X. oryzae* pv. *oryzicola*, a homologue of $Hpa1_{Xoo}$ (Zou *et al*., 2006), predicted two α-helices, ISEKQLDQLLCQ LIQALL and ASPLTQMLNIVGEILQAQ, also at the N- and

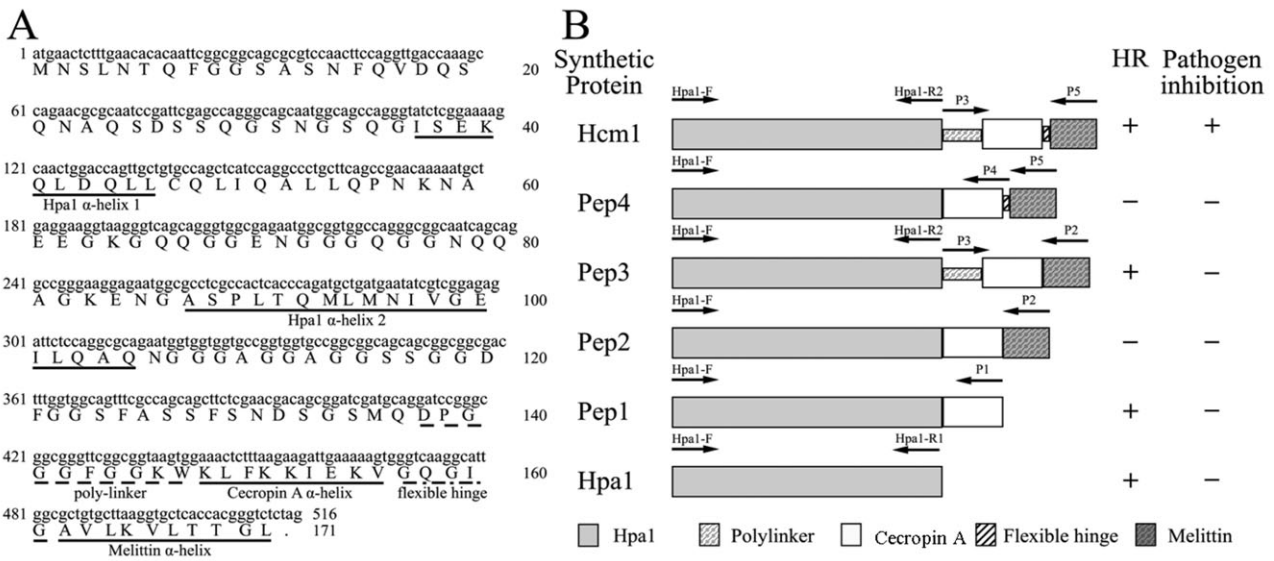

Fig. 1. The coding sequence of Hcm1, the construction of the five chimeric proteins, Pep1, Pep2, Pep3, Pep4 and Hcm1, and results from their biological testing.
A. The DNA and amino acid sequence of Hcm1, indicating the α-helices of Hpa1, cecropin A and melittin (solid underline); the polylinker (dashed underline); and the flexible hinge (dashed-dot underline).
B. Construction of chimeric protein genes with corresponding primer pairs: *pep1* (Hpa1-F/P1); *pep2* (Hpa1-F/P2); *pep3* (the fragment of Hpa1-F/Hpa1-R2 was fused with the DNA of P3/P2 at the BamHI site); pep4 (first amplified by Hpa1-F/P4 and then by Hpa1-F/P5); and *hcm1* (the fragment of Hpa1-F/Hpa1-R2 was fused with the DNA of P3/P5 at the BamHI site). The arrows represent the location and orientation of the primers (Table 2). '+' stands for HR induction in tobacco (*N. tabacum* cv. Xanthi nn) and antimicrobial activity against rice bacterial pathogen *X. oryzae* pv. *oryzicola* by gene products extracted as CFEPs from *E. coli* expression strains containing *pep1*, *pep2*, *pep3*, *pep4* and *hcm1* genes respectively. '–' indicates no HR or no pathogen inhibition. Hpa1 was used as the control.

C-termini (Fig. 1). Since mutations in the N-terminal α-helices of $Hpa1_{Xoo}$ and HpaG of *X. axonopodis* pv. *glycines* led to the loss of HR induction in tobacco (Oh *et al*., 2007; Wang *et al*., 2007; Ji *et al*., 2010), a rational design approach for developing a new antimicrobial protein prompts us to maintain the α-helix structures in Hpa1 while adding other AMPs to its C-terminus. For one of these, cecropin A, the antibacterial activity is due to the α-helix at its N-terminus (Andreu *et al*., 1983; Hancock, 2001; Shai, 2002; Ferre *et al*., 2006; Makovitzki *et al*., 2007); for the second, melittin, the cytolytic and toxic activity towards microbes depends on an α-helix at its C-terminus (Hristova *et al*., 2001; Ferre *et al*., 2006; Glättli *et al*., 2006; Raghuraman and Chattopadhyay, 2007). A hybrid molecule that includes both of these α-helices linked by a flexible hinge, GQGIG, shows high antibacterial activity (Ferre *et al*., 2006; Saugar *et al*., 2006; Xu *et al*., 2007). To investigate whether linkers between Hpa1 and the α-helix of cecropin A, and between the α-helix of cecropin A and the α-helix of melittin, are required for the chimeric proteins to induce HR in plants and to show antimicrobial activity *in vitro*, we constructed five chimeric genes (Fig. 1): *pep1*, where the *hpa1* gene without a stop codon at its 3′ terminus was fused directly to the sequence encoding the α-helix, KLFKKIEKV, of cecropin A, plus a stop codon; *pep2*, where the *pep1* gene was linked directly at its 3′ terminus to the sequence encoding the α-helix, AVLKVLTTGL, of melittin, plus a stop codon; *pep3*, where a polylinker, DPGGFGGKW (Wriggers *et al*., 2005), that maintained the reading frame was used to join the 3′ end of the *hpa1* gene, without a stop codon, to the sequence encoding the α-helices of both cecropin A and melittin (without a flexible hinge between them); *pep4*, where the 3′ terminus of the *hpa1* gene without a stop codon was fused directly (without the polylinker) to the sequence for the hybrid peptide, where cecropin A and melittin were linked via the flexible hinge between them; and *hcm1*, where the polylinker mentioned above linked *hpa1* and the α-helix sequence of cecropin A, and the flexible hinge linked the α-helix sequences of cecropin A and melittin. The unmodified *hpa1* gene was used as the control. The above chimeric genes were cloned with a His-tag sequence into plasmid pET30a(+), producing the recombinant constructs pPep1, pPep2, pPep3, pPep4 and pHcm1 respectively (Table 1, Fig. 1).

Hcm1 exhibits antimicrobial activity

To determine whether the five chimeric proteins described above inhibit bacterial growth, we transferred the plasmids pHpa1, pPep1, pPep2, pPep3, pPep4 and pHcm1 into *Escherichia coli* strain BL21 (DE3), generating expression strains BLHpa1, BLPep1, BLPep2, BLPep3, BLPep4 and BLHcm1 (Table 1) respectively. After induction by IPTG,

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Properties[a]	Source
Strains		
E. coli		
DH5α	F⁻, Φ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *deoR recA endA1 hsdR17*(rK⁻mK⁺) phoA supE44 λ⁻*thi*-l *gyr*A96 *rel*A1	Invitrogen
BL21(DE3)	F⁻, *ompT hsdSB* (rB⁻mB⁻) *gal dcm* (DE3)	Novagen
BLHcm1	Transformant of BL21(DE3) with pHcm1, Km^r	This study
BLPep1	Transformant of BL21(DE3) with pPep1, Km^r	This study
BLPep2	Transformant of BL21(DE3) with pPep2, Km^r	This study
BLPep3	Transformant of BL21(DE3) with pPep3, Km^r	This study
BLPep4	Transformant of BL21(DE3) with pPep4, Km^r	This study
BLHpa1	Transformant of BL21(DE3) with pHpa1, Km^r	This study
B. subtilis		
B168	*trpC2*	This lab
X. oryzae pv. *oryzicola*		
RS105	Wild-type, the causal agent of rice bacterial leaf streak, Rif^r	This lab
R. solanacearum		
ZJ3721	Wild-type, the causal agent of tomato bacterial wilt	Li *et al.* (2010)
P. syringae pv. *tomato*		
DC3000	Wild-type, the causal agent of tomato bacterial spot, Rif^r	This lab
F. graminearum		
ZF21	Wild-type, the causal agent of wheat scab	Zhang *et al.* (2009b)
M. oryzae		
Guy11	Wild-type, the causal agent of rice blast	This lab
A. alternata		
TBA28	Wild-type, the causal agent of tobacco brown spot	This lab
T. cucumeris		
JS01	Wild-type, the causal agent of rice sheath blight	This lab
Plasmids		
pMD18-T	pUC ori, cloning vector, Ap^r	TaKaRa
pET30a (+)	pBR322 origin, F1 origin, *lac*I, His-Tag, S-Tag, Km^r	Novagen
pHcm1	*hcm1* in pET30a(+) at NdeI and XhoI sites, His-tagged, Km^r	This study
pPep1	*pep1* in pET30a(+) at NdeI and XhoI sites, His-tagged, Km^r	This study
pPep2	*pep2* in pET30a(+) at NdeI and XhoI sites, His-tagged, Km^r	This study
pPep3	*pep3* in pET30a(+) at NdeI and XhoI sites, His-tagged, Km^r	This study
pPep4	*pep4* in pET30a(+) at NdeI and XhoI sites, His-tagged, Km^r	This study
pHpa1	*hpa1* in pET30a(+) at NdeI and XhoI sites, His-tagged, Km^r	This study

a. Ap^r = ampicillin resistance; Km^r = kanamycin resistance; Rif^r = rifampicin resistance.

individual cell-free elicitor preparations (CFEPs) of Hpa1, Pep1, Pep2, Pep3, Pep4 and Hcm1 were made from the *E. coli* strains (see *Experimental procedures*). Three microlitres of crudely purified proteins was applied to sterile paper filter discs laid on the surface of NA plates previously inoculated with *X. oryzae* pv. *oryzicola* RS105 as the indicator organism. Inhibition haloes indicating no bacterial growth were seen only around the discs where chimeric protein Hcm1 had been applied and not around those with Hpa1, Pep1, Pep2, Pep3 and Pep4 (Fig. 2A). To rule out the possibility that because of the presence of the α-helices the different fusion proteins might be binding to the *E. coli* membrane, we purified the expressed chimeric proteins from the total membrane preparations of the respective *E. coli* strains before testing. Similar results indicated that only Hcm1 possessed antibacterial activity (results not shown), demonstrating that not only the cecropin A-melittin addition, but also the polylinker between Hpa1 and the α-helix of cecropin A, as well as the flexible hinge between the α-helices of cecropin A and melittin (Fig. 1), are all necessary for antimicrobial activity in Hcm1.

Hcm1 induces the HR in tobacco and activates HR marker gene expression

Since a component of Hcm1 is Hpa1, which triggers the HR in a typical tobacco plant (Zou *et al.*, 2006), we sought to determine whether Hcm1 also elicits the HR in tobacco. We infiltrated into tobacco leaves via needleless syringe the fusion proteins, Pep1, Pep2, Pep3, Pep4 and Hcm1, either as CFEPs or as purified forms (see *Experimental procedures*). All fusion proteins tested, including Hpa1 as the positive control, induced the HR in tobacco except for Pep 2 and Pep 4 (Fig. 2B). These latter two were the only ones that included the α-helices of both cecropin A and melittin (with or without the flexible hinge between them) but did not have the polylinker between Hpa1 and the cecropin A-melittin helices (Fig. 1). These results suggest

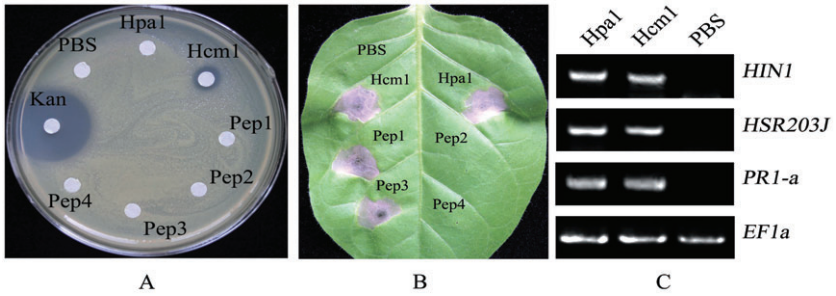

Fig. 2. *In vitro* assays of chimeric proteins for antimicrobial activity against *X. oryzae* pv. *oryzicola* and for HR induction in *N. tabacum* cv. Xanthi nn.

A. Antimicrobial activity of Hpa1, Pep1, Pep2, Pep3, Pep4 and Hcm1 against rice pathogen *X. oryzae* pv. *oryzicola* RS105. Three microlitres of CFEPs of chimeric proteins at approximately 0.1 μM, extracted from the *E. coli* expression strains containing *hpa1*, *pep1*, *pep2*, *pep3*, *pep4* and *hcm1* genes (Table 1), respectively, was added to sterile filter paper discs (0.5 cm diameter), which had been laid on NA plates where 100 μl of *X. oryzae* pv. *oryzicola* RS105 at approximately 1×10^6 cfu ml^{-1} had been spread previously. After 2 days incubation at 28°C, antimicrobial haloes around the discs were recorded. Kanamycin at 10 μg ml^{-1} and PBS buffer were used as the positive and negative controls respectively.

B. Response of tobacco to the chimeric proteins. The CFEPs of Pep1, Pep2, Pep3, Pep4 and Hcm1 at 0.1 μM were infiltrated via needleless syringe into fully expanded leaves of *N. tabacum* cv. Xanthi nn. The HR response was photographed 48 h after infiltration. The CFEP of Hpa1 at 0.1 μM and PBS buffer were used as positive and negative controls respectively.

C. HR marker gene expression was explored by reverse transcription polymerase chain reaction (RT-PCR). Tobacco leaves, infiltrated by the HR-elicitor Hpa1, the chimeric protein Hcm1 (both at 0.1 μM), or PBS buffer, were collected 8 h post infiltration. The same amount of RNA extracted from each sample was used to make cDNA using a TaKaRa RNA PCR Kit (AMV ver. 3.0; TaKaRa). PCR amplifications with *Taq* polymerase were performed using the obtained cDNAs as templates with paired primers (Table 2) of the HR marker genes, *HIN1, HSR203J* and *PR1-a* (Takahashi *et al*., 2004), in tobacco. The obtained PCR products were analysed in 1.2% agarose gels. The *EF1a* gene was used as the internal control to verify the absence of significant variation at the cDNA level in the samples. The above experiments were replicated three times. The results presented are from a representative experiment and similar results were obtained in all other independent experiments.

that the polylinker following Hpa1 is critical to maintaining its HR-inducing activity when it is fused to the α-helices of both cecropin A and melittin, whether or not they have the flexible hinge between them. The data, taken together with those from the antibacterial activity tests, indicate that Hcm1 could be considered a novel chimeric protein that not only inhibits bacterial growth *in vitro*, but also induces the HR *in planta*. In addition, Hcm1 was also a heat-stable protein as Hpa1 seen from the procedure for CFEP preparations.

When the HR is triggered by harpins in tobacco, HR marker genes, such as *HIN1*, *HSR203J* and *PR1-a*, are activated, indicating that SAR occurs via SA signalling (Gopalan *et al*., 1996; Bowling *et al*., 1997; Pontier *et al*., 1998; Takahashi *et al*., 2004). We then investigated whether these HR marker genes were expressed following infiltration via needleless syringe of Hcm1 at 10 μg ml^{-1} into tobacco leaves, while Hpa1 at 10 μg ml^{-1} and PBS buffer were used as the positive and negative controls. At 8 h following infiltration, total RNA was extracted from the treated leaves and reverse transcriptional polymerase chain reaction (RT-PCR) was performed using gene-specific primers for the HR marker genes indicated above (Table 2). The results showed that *HIN1*, *HSR203J* and *PR1-a* were induced by Hcm1 as by Hpa1, suggesting that the chimeric protein Hcm1, like Hpa1, activates plant defence responses (Fig. 2C).

Hcm1 binds the membrane of E. coli *cells*

The yield of Hcm1 from the culture of the *E. coli* expression strain BLHcm1 was less than that of Pep1, Pep2, Pep3 and Pep4 from their respective expression strains, and the colony size of BLHcm1 on Luria–Bertani (LB) plates was smaller than that of BLPep1, BLPep2, BLPep3 and BLPep4 (data not shown). This suggested that Hcm1 may be toxic to *E. coli*, possibly through binding to the membrane of the bacterium, since the α-helix structures in Hpa1, cecropin A and melittin have that property (Hristova *et al*., 2001; Lee *et al*., 2001; Shai, 2002; Allende *et al*., 2005; Racapé *et al*., 2005; Raghuraman and Chattopadhyay, 2007). To test this, we used Hcm1 and Hpa1 from the BLHcm1 and BLHpa1 strains, respectively, in two forms: the CFEPs from lysed cells plus heat treatment at 100°C for 10 min, and the preparations from membrane fragments purified through a HisTrapTM^{FF} column (see *Experimental procedures*). Analysis by SDS-PAGE, followed by immunoblotting using a polyclonal anti-Hpa1 antiserum, showed the larger size expected for Hcm1 from either source (19.5 kD as compared with 13.6 kD for Hpa1), but lower amounts of Hcm1 and Hpa1 from the cells of the expression strains than from the membrane fragments (Fig. 3A). Thus, Hcm1, as Hpa1, may bind to the bacterial cell membrane, but Hpa1 is not toxic to the bacterial hosts.

Table 2. Primers used in this study.

Primers	5′- to 3′-sequence, restriction sites underlined	Description
Hpa1-F	AACATATGATGAACTCTTTGAACAC	414 bp *hpa1* gene
Hpa1-R1	TTCTCGAGTTACTGCATCGATCCGCTGTCG	XhoI site was added at 3′-termi of *hpa1*
Hpa1-R2	TTGGATCCTTACTGCATCGATCCGCTGTCG	BamHI site was added at 3′-termi of *hpa1*
P1	CTCGAGCTACACTTTTTCAATCTTCTTAAAGAGTTTCTGCATCGAT	Sequence for KLFKKIEKV was fused to C-terminus of Hpa1
P2	AACTCGAGCTAGAGACCCGTGGTGAGCACCTTAAGCACAGCGACT	Sequence for AVLKVLTTGL was fused to C-terminus of Hpa1
P3	CAGGATCCGGGCGGCGGGTTCGGCGGTAAGTGGAAACTCTTTAAG	Sequence for the polylinker DPGGGFGGKW was fused before the α-helix of cecropin
P4	TTGACCCACTTTTTCAATCTTCTTAAAGAG	Squence for the flexible hinge GQGIG was fused after the α-helix of cecropin
P5	AACTCGAGCTAGAGACCCGTGGTGAGCACCTTAAGCACAGCGCCA	Sequence for the α-helix of melittin was fused after the flexible hinge
Hin1-F	GAACGGAGCCTATTATGGCCCTTCC	867 bp *HIN1* gene
Hin1-R	CATGTATATCAATGAACACTAAACGCCGG	
HSR203J-F	TTGAACACACAATTCGGCGG	618 bp *HSR203J* gene
HSR203J-R	TTACTGACTCGATGCGCTGTC	
PR1-a-F	GGCGTTCTCTTTTCACAATTGCCTTCAT	495 bp *PR1-a* gene
PR1-a-R	AACGGACTTTCGCCTCTATAATTACCTG	
EF1a-F	AGACCACCAAGTACTACTGCAC	495 bp *EF1a* gene
EF1a-R	CCACCAATCTTGTACACATCC	

To verify our earlier results using the CFEPs (Fig. 2), that the antibacterial activity of Hcm1 is due to the addition of the cecropin A-melittin hybrid to the Hpa1 backbone, we used purified Hcm1 to test for growth inhibition of *E. coli* on LB plates, using Hpa1 at 5 μM as a negative control and kanamycin (Km) at 10 μg ml^{-1} as a positive control. Indeed, the application of 0.5 μM of Hcm1 to a sterile paper disc caused an obvious inhibition halo against *E. coli* BL21 (DE3), while Hpa1 did not (Fig. 4A), confirming that the addition of the cecropin A-melittin hybrid at the C-terminus of Hpa1 confers antibacterial activity to Hcm1.

Hcm1 is susceptible to proteolysis

Food safety concerns require that AMPs designed for agricultural use be susceptible to proteolysis in nature. Thus, we compared the susceptibility to protease K digestion of the chimeric protein Hcm1 with that of Hpa1, since harpins are being used currently in agriculture (Dong *et al*., 1999; Fontanilla *et al*., 2005; Zhao *et al*., 2006; Chen *et al*., 2008; Shao *et al*., 2008). Equivalent amounts of purified Hcm1 and Hpa1 (at 10 μM) were incubated with protease K (1 U μl^{-1}) at room temperature for 75 min and the degradations were monitored by an Easy Protein

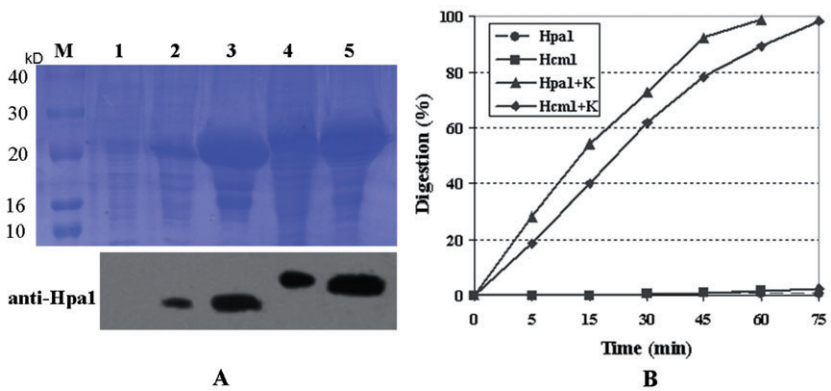

Fig. 3. Expression and purification of Hcm1 detected by immunoblotting analysis (A) and Hcm1 susceptibility to protease K (B). The CFEPs of Hpa1 (Lane 2) and Hcm1 (Lane 3), and the purified Hpa1 (Lane 4) and Hcm1 (Lane 5) (see *Experimental procedures*) from BLHpa1 containing the *hpa1* gene and BLHcm1 harbouring the *hcm1* gene, respectively, were detected by SDS-PAGE (6% stacking gel, 12% separation gel) (upper panel) and analysed by immunoblotting using anti-Hpa1 rabbit IgG as the primary antibody. BL21 (DE3) with empty vector pET30a(+) (Lane 1) was used as the control. To test protease susceptibility, purified Hpa1 and Hcm1 at 10 μM were incubated with 1 U of protease K (Hpa1+K and Hcm1+K) at room temperature for 0, 5, 10, 15, 30, 45, 60 and 75 min, and the digestion was monitored using a protein quantification kit (TransGen Biotech, Beijing, China) on a Thermo NANODROP 1000 Spectrophotometer. Treatment without protease K was used as the control (Hpa1 and Hcm1). Three independent experiments were performed and similar results were obtained.

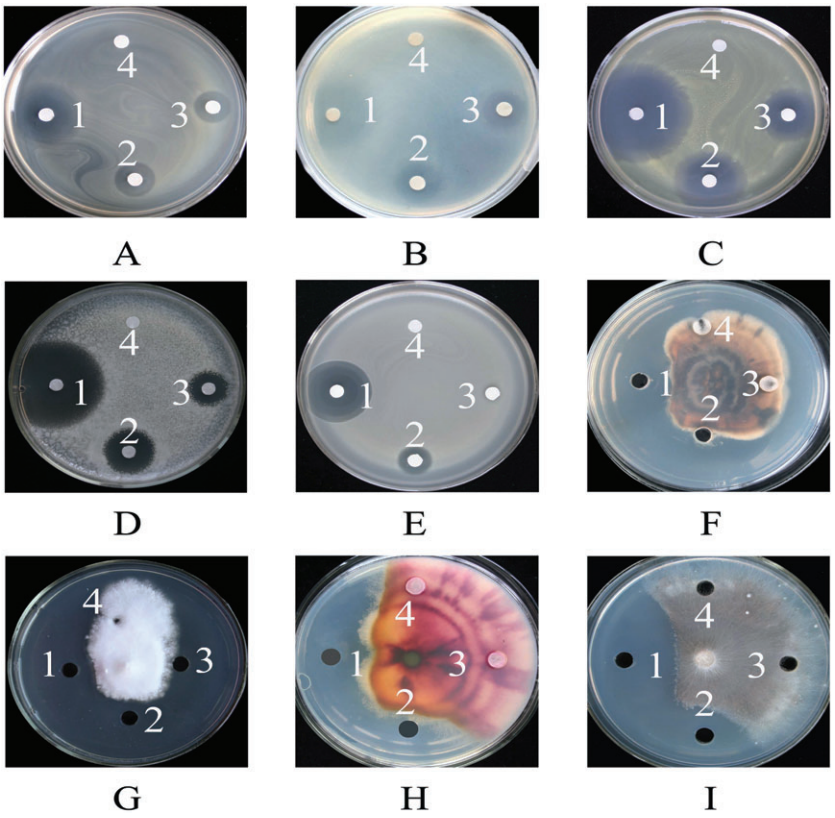

Fig. 4. Antimicrobial activity of Hcm1 against bacteria and fungi on agar plates. 1. Kanamycin (10 μg ml^{-1}) for bacteria and carbendazim (50 μg ml^{-1}) for fungi were used as positive controls; 2. Hcm1 (5 μM); 3. Hcm1 (1 μM); 4. Hpa1 (5 μM). The sterile filter paper discs (5 mm diameter) were dipped in the above solutions, respectively, and then laid on NA plates where 100 μl of the respective bacteria (below) at approximately 1×10^6 cfu ml^{-1} had been spread previously. After 2 days incubation at 28°C, antimicrobial haloes around the discs were recorded. For antifungal tests, 5-mm-dia mycelial discs of the fungi listed below were placed on PDA plates, and then 10 μl of the above solutions were added to 5-mm-dia holes made around the mycelial discs by a hole punch. Antimicrobial haloes around the holes were recorded after 5 days at 28°C, depending on different fungal growth. Similar results were obtained from three replications. (A) *E. coli* BL21 (DE3); (B) *R. solanacearum* ZJ3721; (C) *X. oryzae* pv. *oryzicola* RS105; (D) *P. syringae* pv. *tomato* DC3000; (E) *B. subtilis* B168; (F) *A. alternata* TBA28; (G) *M. oryzae* Guy11; (H) *F. graminearum* ZF21; (I) *T. cucumeris* JS01.

Quantitative Kit (TransGen Biotech, Beijing, China) on a Thermo NANODROP 1000 Spectrophotometer over time. Notably, 100% of Hcm1 was degraded by protease K in 75 min, indicating that Hcm1 is also susceptible to proteolysis, but less so than Hpa1, which required only 60 min for 100% digestion (Fig. 3B).

Hcm1 shows broad spectrum inhibition of microbial growth

We next tested the antimicrobial activity of Hcm1 against a broad spectrum of microbes. We chose as test organisms: *E. coli* BL21 (DE3) as a Gram-negative non-pathogenic bacterium; *Bacillus subtilis* B168 as a Gram-positive non-pathogenic bacterium; *X. oryzae* pv. *oryzicola* RS105, *R. solanacearum* ZJ3721 and *Pseudomonas syringae* pv. *tomato* DC3000 as Gram-negative pathogenic bacteria; and *Magnaporthe oryzae* Guy11 (causal agent of rice blast), *Fusarium graminearum* ZF21 (causal agent of wheat scab), *Alternaria alternata* TBA28 (causal agent of tobacco brown spot) and *Thanatephorus cucumeris* JS01 (causal agent of rice sheath blight) as plant pathogenic filamentous fungi (Table 1). Hcm1 at 5 μM on sterile discs produced inhibition haloes against not only the bacteria, but also the fungi, whereas Hpa1 at the same concentration did not (Fig. 4). At a lower concentration (1 μM), Hcm1 showed no antimicrobial activity against *B. subtilis* (Fig. 4E), *F. graminearum* (Fig. 4H) and *T. cucumeris* (Fig. 4I). These results indicate that Hcm1 shows broad spectrum antimicrobial activity.

The α-helix structures of Hpa1, cecropin A and melittin are essential for binding to and/or forming pore-like structures in targeted cell membranes (Hristova *et al.*, 2001; Ferre *et al.*, 2006; Glättli *et al.*, 2006; Oh *et al.*, 2007; Wang *et al.*, 2007; Ji *et al.*, 2010). This prompted us to investigate whether Hcm1 would inhibit fungal spore germination. For this, we mixed spores of *M. oryzae* Guy11 or *F. graminearum* ZF21 with Hcm1 at a final concentration of 5 μM, and used Hpa1 at 5 μM as the control. We incubated the mixtures at 25°C and assessed spore germination status at 0, 4 and 8 h using bright-field microscopy. We observed that fungal spores of either *M. oryzae* or *F. graminearum* could germinate and form germ tubes 4 h post incubation when incubated with Hpa1, but not when treated with Hcm1. At 8 h, we observed hyphae extending from the germ tubes in the Hpa1-treated samples, while at the same time in the Hcm1-treated samples we only rarely observed even germ tube formation (Fig. 5). The data here suggest that the inhibition of fungal spore germination by Hcm1 is due to the addition of the cecropin A-melittin hybrid peptide at the C-terminus of Hpa1. This is consistent with our previous observation that Hcm1 shows broad antimicrobial activity (Figs 2 and 4).

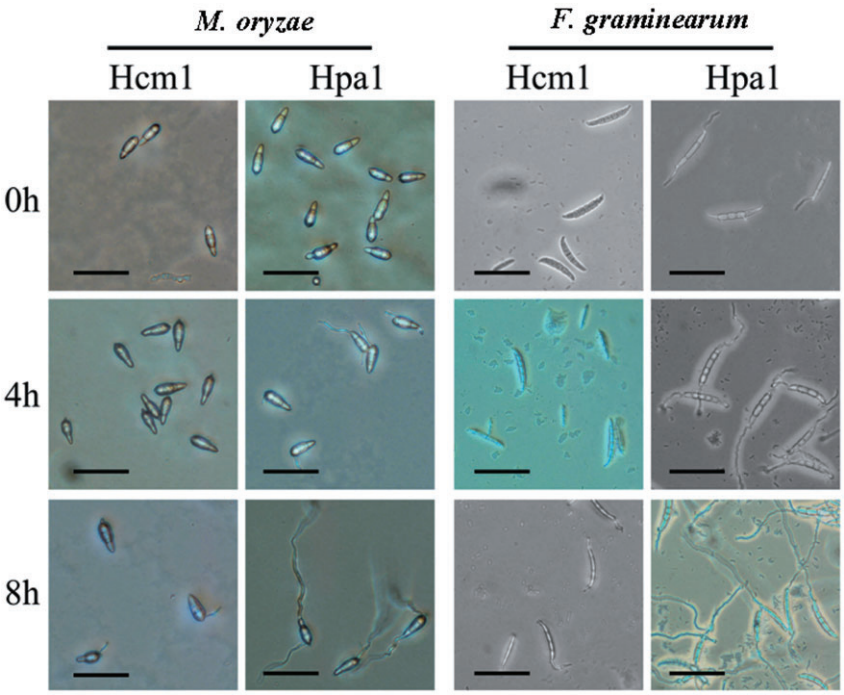

Fig. 5. Inhibition of fungal spore germination by the chimeric protein Hcm1. Fresh spore suspensions (1×10^3 spore μl^{-1}) of *M. oryzae* and *F. graminearum* were treated with Hcm1 or Hpa1 (5 μM). Hpa1 was used as the negative control. Spore germination was observed under microscopy (100×) at 0, 4 and 8 h following treatment. The experiment was replicated three times. Scale bar = 50 μM.

To more precisely define the antimicrobial activity of Hcm1, we assessed the 50% effective dose (ED_{50}) values for Hcm1 with representative microbes (Table 3). Hcm1 inhibited the growth of all nine bacteria and fungi tested. The ED_{50} values for Hcm1 ranged from 0.25 to 1.25 μM for the bacteria tested, and from 1.25 to 5 μM for the following fungi: *M. oryzae*, *F. graminearum*, *T. cucumeris* and *A. alternate* (Table 3). The results indicated that the Gram-negative bacteria tested were more sensitive to Hcm1 than was the Gram-positive one, and that the bacteria were generally more sensitive to Hcm1 than were the fungi.

The minimum inhibitory concentrations (MICs) determined for Hcm1 using the same microbes were consistent with the ED_{50} values. In general, Gram-negative bacteria were more sensitive to Hcm1 than were Gram-positive bacteria or plant pathogenic filamentous fungi (Table 3). Interestingly, the germination of *M. oryzae* spores was completely suppressed by Hcm1 at a MIC of 2.5–3 μM, lower than that determined for *F. graminearum*, *T. cucumeris* and *A. alternate* (Table 3).

Table 3. Antimicrobial activity of Hcm1 against *E. coli* and plant-pathogenic bacteria and fungi.

Pathogens	ED_{50} (μM)	MIC (μM)
E. coli DH5α	0.5	0.75–1
X. oryzae pv. *oryzicola* RS105	0.5–0.75	1–1.25
P. syringae pv. *tomato* DC3000	0.25–0.5	1
R. solanacearum ZJ3721	0.75	1–1.25
B. subtilis B168	1.25	2–2.5
F. graminearum ZF21	2.5	3.5–4
T. cucumeris JS01	2–2.5	3.5
M. oryzae Guy11	1.25–1.5	2.5–3
A. alternata TBA28	4–5	6–7.5

Since the Hcm1 fusion protein showed similar antibacterial activity against the Gram-negative plant pathogenic bacteria tested, we generated a survival time-course for mid-logarithmic-phase culture suspensions of *X. oryzae* pv. *oryzicola*, *P. syringae* pv. *tomato* and *R. solanacearum* treated with Hcm1 at 2 μM. In this test, *R. solanacearum* survived longer than did *X. oryzae* pv. *oryzicola* and *P. syringae* pv. *tomato*, the latter being the most sensitive of the three; the control Hpa1 had no effect on the survival of these bacteria (Fig. 6), implying that the addition of the cecropin–melittin hybrid at the C-terminus of Hpa1 confers the antimicrobial activity to Hcm1.

Treatment of plants with Hcm1 induces resistance to fungal, bacterial and viral infection

Since Hcm1 activates plant defence genes *in planta* (Fig. 2) and shows antimicrobial activity *in vitro* (Figs 2, 4–6), we investigated whether Hcm1 reduces plant disease severity by spraying it on plants prior to inoculation with plant pathogens. To test different types of pathogens on different plants, we chose tobacco mosaic virus (TMV), the bacterium *R. solanacearum* and the fungus *M. oryzae*, which cause the diseases tobacco mosaic, bacterial wilt of tomato, and rice blast respectively. We investigated whether prior treatment of plants with Hcm1 can: (i) increase resistance to TMV in *Nicotiana tabacum* cv. Xanthi nn, which does not contain the *N* gene and

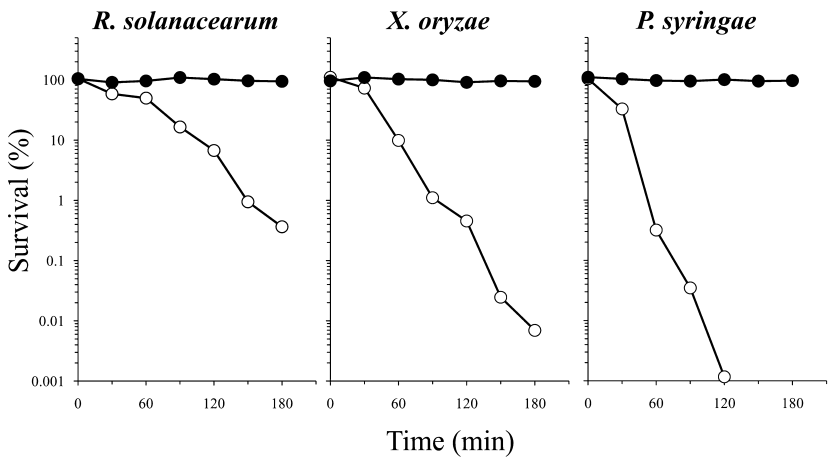

Fig. 6. Kinetics of survival of *R. solanacearum* ZJ3721, *X. oryzae* pv. *oryzicola* RS105 and *P. syringae* pv. *tomato* DC3000 in the presence of 2 μM Hcm1 for 3 h. Viable cells were determined at the times indicated. Incubation was at 28°C and in NB for *R. solanacearum* and *X. oryzae* pv. *oryzicola, or in* Kings B broth for *P. syringae* pv. *tomato*. Treatment was with Hcm1 (white circles) or with Hpa1 (2 μM), the negative control (black cirlces).

produces HR-like necrotic-like lesions when TMV is rubbed on leaves (Enyedi *et al.*, 1992; Ehrenfeld *et al.*, 2008); (ii) reduce the number of brown necrotic spots caused when *M. oryzae* Guy11 infects seedlings of *Oryza sativa* cv. CO-39 (Zhang *et al.*, 2009a); and (iii) reduce the bacterial wilt seen in *Solanum lycopersicum* cv. Suhong 2003 when infected by *R. solanacearum* ZJ3721 (Li *et al.*, 2010). Ten plants each of 2-month-old tobacco, 1-month-old rice and 1-month-old tomato were sprayed fully with Hcm1 or Hpa1 (1.5 μM), and then sprayed again 3 days later. Three days after the second spraying, the plants were inoculated – the tobacco by softly rubbing the leaf surfaces with cotton tips containing a TMV-emery powder mixture, the rice by spraying fresh *M. oryzae* spores (1×10^5 spore ml^{-1}) on the leaf surfaces, and the tomato by injecting *R. solanacearum* (1×10^8 cfu ml^{-1}) into the stems with needled syringes. Disease symptoms were assessed at different times following inoculation as indicated in the legend to Fig. 7. The number of necrotic spots and the necrotic area per leaf were measured for the tobacco plants inoculated with TMV and the rice plants inoculated with *M. oryzae* (which produced brown dark

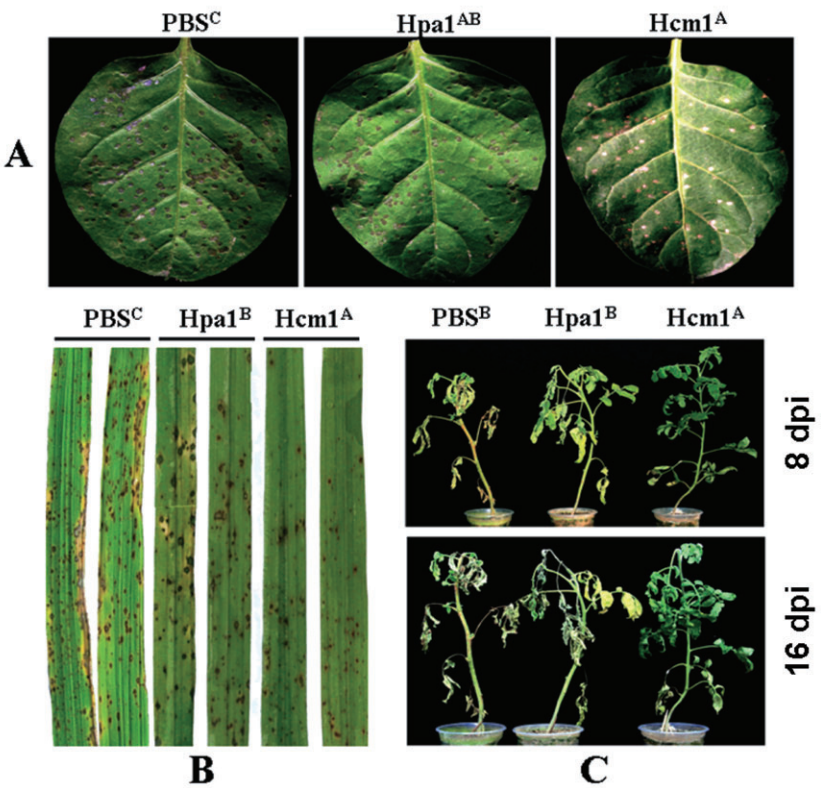

Fig. 7. Plant disease reduction by Hcm1.
A. Hcm1 induces resistance to tobacco mosaic virus (TMV) infection. Three days after spraying *N. tabacum* cv. Xanthi nn plants with Hcm1 (1.5 μM), cotton swabs were used to gently and evenly apply freshly prepared TMV inoculum mixed with emery powder (see *Experimental procedures*) to the tobacco leaf surfaces. Necrotic spots formed by TMV were counted 3 days post inoculation (dpi).
B. Application of Hcm1 to prevent rice blast caused by *M. oryzae*. Three days after the spraying of Hcm1 at 1.5 μM, a suspension of *M. oryzae* Guy11 spores (1×10^5 spore ml^{-1}) was sprayed on leaves of 4-week-old *O. sativa* cv. CO-39. The necrotic lesions of rice blast were counted 5 dpi.
C. Prevention of tomato bacterial wilt disease by Hcm1. A fresh *R. solanacearum* suspension was adjusted to 1×10^8 cfu ml^{-1} and needle-injected into stems of one-month old seedling *S. lycopersicum* Suhong 2003 three days after spraying with Hcm1. The percentage of wilted leaves per plant was determined 8 and 16 dpi respectively. PBS buffer and Hpa1 at 1.5 μM were used as the negative and positive controls. The experiments shown were replicated three times and similar results were obtained. The different letters at the shoulders of PBS, Hpa1 and Hcm1 indicate significant differences in reducing disease severities at $P = 0.01$ by *t*-test.

Table 4. Statistical analyses of disease index (DI) and inhibition rate for three plant pathogens on plants pre-treated with Hcm1.

Plant disease	Treatment	Necrotic spots	Necrotic area per leaf (%)	Wilt rate (%)[1]	DI[2]	IR (%)[3]
Tobacco mosaic	Hcm1	98.5 ± 19.49^{A}*	24.1 ± 6.61^{A}	ND	55.73^{A}	46.43
	Hpa1	151.6 ± 30.98^{AB}	28.5 ± 6.97^{AB}	ND	61.42^{AB}	35.71
	PBS	258.4 ± 63.15^{C}	45.3 ± 7.71^{C}	ND	93.33^{C}	–
Rice blast	Hcm1	48.5 ± 11.37^{A}	13.3 ± 3.38^{A}	ND	48.32^{A}	47.28
	Hpa1	95.6 ± 13.24^{B}	38.2 ± 6.94^{B}	ND	84.02^{B}	5.53
	PBS	105.3 ± 15.68^{C}	41.4 ± 3.57^{C}	ND	91.22^{C}	–
Tomato bacteria wilt	Hcm1	ND	ND	74.8 ± 8.52^{A}	55.13^{A}	38.89
	Hpa1	ND	ND	92.7 ± 5.94^{B}	83.32^{B}	7.41
	PBS	ND	ND	96.7 ± 4.87^{B}	90.27^{B}	–

*Data are the mean ± standard deviation of triplicate measurements. The different letters in each data column indicate significant differences at $P = 0.01$ by *t*-test. The experiment was repeated three times and similar results were obtained. ND = not done.
[1]Wilt rate equals percentage of leaves wilted per plant (see *Experimental procedures*).
[2]DI means disease index (see *Experimental procedures*).
[3]IR presents inhibition rate (see *Experimental procedures*).

spindle spots), and the severity of plant wilting was assessed for tomato bacterial wilt. The data in Fig. 7 and Table 4 show clearly the effectiveness of Hcm1-induced resistance against TMV, *M. oryzae* and *R. solanacearum* infections. The control plants initially sprayed with PBS showed significantly more infection by all three pathogens than the Hcm1-treated plants (Table 4). Prior application of Hpa1, which had been shown previously to induce systemic resistance against plant pathogen infections (Fontanilla *et al*., 2005; Zhao *et al*., 2006; Shao *et al*., 2008), also significantly reduced the number of necrotic spots produced by TMV or *M. oryzae* in our experiments, but it was not as effective as Hcm1 in reducing the incidence of bacterial wilt in tomato (Fig. 7, Table 4). The significantly better ($P = 0.01$, *t*-test) protection seen with prior treatment with Hcm1 versus Hpa1 against both rice blast and bacterial wilt in tomato (Fig. 7D, Table 4) may be due to the addition in Hcm1 of the cecropin A-melittin hybrid peptide at its C-terminus. This suggests that application of the rationally designed protein Hcm1 to crop plants may be a new and effective way to control plant diseases.

Discussion

Following years of work on the *de novo* and rational design of novel AMPs for use as drugs to fight disease in both agriculture and medicine, there is now increasing interest in creating chimeric or hybrid fusions between different molecules with antimicrobial activity and other properties (Yevtushenko *et al*., 2005; Yevtushenko and Misra, 2007; Melo *et al*., 2009). However, there is to date no work that shows that an HR-elicitor can be fused to active domains of AMPs and make chimeric proteins that show both HR induction and antimicrobial activity in plants. In the present study, we created five chimeric proteins, all of which contained the entire HR-elicitor Hpa1 of the rice pathogen *X. oryzae* pv. *oryzicola*, and which had the active antimicrobial domains of cecropin A and/or melittin linked to Hpa1 with or without the polylinker, and with or without the flexible hinge between the two antimicrobial domains. The crude CFEPs of all five constructs were tested for their abilities to inhibit *in vitro* the plant pathogenic bacterium *X. oryzae* pv. *oryzicola* and to induce the HR in tobacco. We found that only one of these, Hcm1, both induced the HR in tobacco and inhibited bacterial growth. This indicates that both the polylinker between Hpa1 and the cecropin A-melittin hybrid, as well as the flexible hinge between the α-helices of cecropin A and melittin, are necessary for these two activities (Fig. 1). The polylinker may stabilize the two α-helix structures of Hpa1, which in that protein are essential for HR induction, amyloidogenesis and pore-like formation in plants (Oh *et al*., 2007; Wang *et al*., 2007; Ji *et al*., 2010), thus protecting it from destabilizing interactions with the two α-helices in the cecropin A and melittin domains. The flexible hinge in the middle of the cecropin A-melittin hybrid, on the other hand, is necessary for Hcm1 to exhibit pesticidal activities, consistent with earlier studies on the synthetic hybrid of cecropin A-melittin (Ferre *et al*., 2006; Saugar *et al*., 2006; Xu *et al*., 2007). The purified Hcm1 exhibited antimicrobial activity against prokaryotic Gram-negative and Gram-positive bacteria and eukaryotic fungi (Figs 4–6), and it not only induced the HR in tobacco but also activated plant defence genes possibly through a SA signalling pathway, resulting in reduced infections by viral, bacterial and fungal pathogens (Figs 2 and 7). These observations mark the successful creation of functional Hcm1 and offer a new strategy for drug design to control plant, and possibly animal, diseases, where combining in a single molecule a plant defence activator with one or more available AMPs generates a synergism in plant protection.

The chimeric protein Hcm1 triggered the HR and activated the expression of HR marker genes in tobacco, e.g.

HIN1, *HSR203J* and *PR1-a* (Takahashi *et al*., 2004), both activities typical of harpins (Desikan *et al*., 1998; 1999; Dong *et al*., 1999; Xie and Chen, 2000; Clarke *et al*., 2005), like Hpa1, which are secreted by plant pathogenic bacteria (Zou *et al*., 2006). In addition, TMV infection efficiency was reduced following the spraying of Hcm1 onto tobacco plants to almost the same extent as with Hpa1 (Table 4, Fig. 7), suggesting that the chimeric protein may benefit plants through the induction of SAR. Moreover, protective effects were greater for Hcm1 than for Hpa1 following spraying of tomato and rice with Hcm1 to protect against tomato bacterial wilt, caused by *R. solanacearum*, and rice blast, caused by *M. oryzae*, possibly due to the antimicrobial activity shown uniquely by Hcm1. Thus, the cecropin A-melittin hybrid that was fused to the C-terminus of Hpa1 in the creation of Hcm1 may contribute to the inhibition of bacterial and fungal infections in plants, in a similar manner to the synergistic effects on plant protection seen with the defence activator benzothiadiazole and chemical fungicides (Molina *et al*., 1998).

To the best of our knowledge, it has not been reported that a chimeric protein made by joining a harpin (e.g. Hpa1, an HR-elicitor) via a polylinker to the cecropin A-melittin hybrid is effective as a bactericidal and fungicidal agent against plant pathogens. The effective inhibitory concentrations of the chimeric protein varied significantly for different bacteria and fungi (Table 3). In a typical MIC assay, we found that the Gram-negative bacteria *E. coli* BL21 (DE3), *X. oryzae* pv. *oryzicola* RS105, *P. syringae* pv. *tomato* DC3000 and *R. solanacearum* ZJ3721 are more sensitive to Hcm1 than is the Gram-positive bacterium *B. subtilis* B168, whereas the fungus *M. oryzae* Guy11 is more sensitive to Hcm1 than is *F. graminearum* ZF21, followed by *T. cucumeris* JS01 and then *A. alternata* TBA28 in decreasing sensitivities (Table 3). These differing susceptibilities of bacteria and fungi to Hcm1 may be attributed to variation in the components outside the plasma membranes of the target microbes. These include for bacteria not only the thickness of the cell wall but also the charge and lipid composition of membranes, and for fungi the thickness and composition of their cell walls, all of which can influence the rate by which cationic peptides bind to the plasma membrane (Marcos *et al*., 2008). It is possible that a thin layer of peptidoglycan outside Gram-negative bacteria, as opposed to the thicker layer in Gram-positive bacteria, provides less of a barrier to Hcm1's binding to the plasma membrane inside. A similar situation may be true for the thick cell walls of fungal spores, made of chitin and other polysaccharides. In fact, the thickness of the *M. oryzae* spore is less than that of *F. graminearum* or *T. cucumeris*, and much less than that of *A. alternata* (Carlile *et al*., 2001). The inhibition of fungal spore germination (Fig. 4) may be due to a mechanism of antimicrobial activity by Hcm1 which involves first the so-called 'self-promoted uptake' across the membrane, after which the cationic portion of Hcm1 interacts with the negatively charged phospholipids of the membrane, followed by either channel formation or simple membrane disruption (Hristova *et al*., 2001; Shai, 2002; Allende *et al*., 2005; Marcos *et al*., 2008). By such a mechanism, Hcm1 may first lie parallel to the surface of the phospholipid bilayer, with its hydrophobic sides facing the membranes and its cationic sides facing outward, until a threshold concentration is reached. The purification of our chimeric Hcm1 from the membrane fraction of the *E. coli* expression strain (Fig. 3) supports this hypothesis. Thus, in designing a chimeric HR-elicitor which also contains active domains of AMPs, maintaining the helix-forming capacity may be even more important. To explore this further, we are currently working on an alternative design where the cecropin A-melittin hybrid peptide is linked to Hpa1 at its N-terminus. A recent study demonstrates that a 24-amino-acid peptide of HrpZ in *P. syringae* is the HR-elicitor domain (Haapalainen *et al*., 2011), suggesting that this could be substituted for the full-length Hpa1 in Hcm1. On the other hand, the HR-activator domain of Hpa1 needs to be explored fully to improve this particular Hcm1 molecule as a drug for crop protection.

Because of the high production costs of synthetic AMPs (Marcos *et al*., 2008), we turned instead to expression in an *E. coli* heterologous system. Genetically modified *E. coli*, and possibly other microbes, like a *B. subtilis* biocontrol agent (whose engineering we attempted but failed), could produce suitable amounts of the chimeric protein Hcm1 (Fig. 2) to add to a plant protection formulation as a fungicidal and bactericidal agent (Table 4, Fig. 7) that could be used as a spray treatment at the time of disease threat, before crop harvesting. It is noteworthy that the AMP Hcm1 could be successfully over-produced in *E. coli* without killing it. This could possibly be due to its compartmentalization in inclusion bodies or an inactive conformation when inside bacterial cells, or to the inability of the peptide to insert into the bacterial plasma membrane from the inside of the cell. Recent evidence from studies in harpin- and AMP-producing transgenic plants (Fontanilla *et al*., 2005; Yevtushenko *et al*., 2005; Sohn *et al*., 2007; Chen *et al*., 2008; Shao *et al*., 2008) leads us to assume that the chimeric *hcm1* gene could be used in transgenic plants, possibly under a pathogen-induced promoter rather than a constitutive one (Bolton, 2009), not only for plant protection against pathogen infection, but also as a model to explore the modulation of the properties of these fusion genes through sequence modification.

Although the possibility of undesirable toxic effects caused by Hcm1 to other living organisms, including beneficial bacteria and mycorrhizal fungi associated with plants, needs to be fully investigated, Hcm1 is sensitive to

protease digestion (Fig. 3) and thus should not accumulate in the environment. While stability to protease digestion is generally a desirable property in AMPs in order to assure a reasonable half-life, this must be balanced with a degree of protease sensitivity in order to address environmental safety and avoid its build-up in the environment. Because the chimeric protein Hcm1 is sensitive to protease K *in vitro* (Fig. 3), as is the synthetic hybrid of cecropin A-melittin (Ferre *et al*., 2006), it is likely that proteases from epiphytic microorganisms or intrinsic to plant tissues will degrade Hcm1. Engineered sequence changes to cloned AMPs have been shown to enhance desirable properties (Andreu *et al*., 1983; Cavallarin *et al*., 1998; Ferre *et al*., 2006; Saugar *et al*., 2006), offering a strategy to further improve rationally designed drugs. Our demonstration that the chimeric Hcm1 can both induce plant defence responses and directly inhibit microbial growth makes it a very promising candidate for both protecting plants from plant disease, and thus improving crop yields, while also ensuring environmental and food safety.

Experimental procedures

Strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The wild-type *X. oryzae* pv. *oryzicola* RS105 and *R. solanacearum* ZJ3721 were grown in NA (0.5% peptone, 0.1% yeast extract, 1% sucrose, 0.3% beef extract and 1.5% agar), or NB (NA without agar) medium at 28°C. *Escherichia coli* and *B. subtilis* strains grow in LB (0.5% yeast extract, 1% tryptone, 1% NaCl, with or without 1.5% agar) at 37°C. *Pseudomonas syringae* pv. *tomato* DC3000 was grown in Kings B medium (20% Peptone, 1.5% K_2HPO_4, 1.5% $MgSO_4{\cdot}7H_2O$, 1.5% agar, pH 7.2) at 30°C. All of the plant pathogenic fungi, including *F. graminearum* ZF21, *M. oryzae* Guy11, *A. alternata* TBA28 and *T. cucumeris* JS01, were grown in PDA (20% potato extract, 2% glucose, 1.8% agar) at 28°C. Unless otherwise specified, antibiotics were used at the following concentrations when required: ampicillin (Ap) at 100 μg ml^{-1}, Km at 50 μg ml^{-1}, rifampicin (Rif) at 50 μg ml^{-1}.

Plant growth conditions

Nicotiana tabacum cv Xanthi nn (producing HR-like necrosis when tobamovirus TMV infects) (Ehrenfeld *et al*., 2008), *O. sativa* cv. CO-39 and *S. lycopersicum* cv. Suhong 2003 plants were grown and maintained under greenhouse conditions (50% humidity, 25–28°C) for different periods of time, depending on the purpose of the experiment, before being used for Hcm1 application and/or pathogen inoculation as described elsewhere. To satisfy the conditions for rice blast development, the rice plants inoculated with *M. oryzae* were moved into a chamber as described by Zhang and colleagues (2009a).

DNA manipulation and plasmid construction

DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, subcloning, electroporation, PCR, and Southern and Western blot analyses were performed according to standard procedures (Sambrook and Russell, 2001). The PCR primers used are listed in Table 2. All PCR products used in cloning were first cloned into the pMD18-T vector (Table 1) and verified by sequencing (Takara, Dalian, China). DNA sequences were analysed with VECTOR NTI software (http://www.invitrogen.com).

To clone a 414 bp *hpa1* gene (Zou *et al*., 2006) from the genome of *X. oryzae* pv. *oryzicola* strain RS105 (Table 1), the primers Hpa1-F/Hpa1-R1 (Table 2) were used. The *hpa1* PCR product was used as the first template in the generation of *pep1* (Fig. 1B) with the primers Hpa1-F/P1 (Table 2), where the active domain, KLFKKIEKV, of cecropin A was directly fused at the C-terminus of Hpa1. This PCR product was later used as the template to synthesize *pep2* (Fig. 1A) with primers Hpa1-F/P2 (Table 2), in order to add the active domain, AVLKVLTTGL, of melittin to Pep1 at its C-terminus. The PCR products, *hpa1*, *pep1* and *pep2*, were ligated into pET30a(+) (Novagen, USA) using the NdeI and XhoI sites, producing the constructs pHpa1, pPep1 and pPep2 respectively (Table 1).

Since the active domains of cecropin A and melittin are α-helix structures (Ferre *et al*., 2006; Saugar *et al*., 2006) that could potentially interact with the structure of Hpa1 in a hybrid protein and thus affect Hpa1's ability to elicit the HR in tobacco, a polylinker, DPGGGFGGKW (Wriggers *et al*., 2005), was used to fuse Hpa1 with the active domains of cecropin A and melittin at BamHI sites. To do so, the primers P3/P2 (Table 2, Fig. 1A) were used to amplify the sequence encoding the polylinker and the active domains of cecropin A and melittin, using the *pep2* gene as the template. Then the full sequence of the *hpa1* gene was added via the BamHI site at C-terminus of Hpa1, without a stop code, by PCR amplification with the primers Hpa1-F/Hpa1-R2 (Table 1, Fig. 1B). These two fragments were ligated together at the BamHI site and the linkage was used as the template to PCR-amplify the *pep3* gene with the primers Hpa1-F/R2 (Table 1, Fig. 1B). The amplified DNA was ligated into pET30a(+) at the NdeI and XhoI sites, giving the constructs pPep3 (Table 1).

In order to maintain the antimicrobial activity associated with the α-helix structures of the active domains of cecropin A and melittin, a flexible hinge GQGIG (van Noort *et al*., 2004) was added between those two domains with the primer pairs Hpa1-F/P4 and Hpa1-F/P5 (Table 1, Fig. 1B), respectively, by using the *pep1* gene as the template. The final PCR product was ligated into pET30a(+) at NdeI and XhoI sites, producing pPep4.

To avoid perturbing the α-helix domains found in Hpa1, at the N-terminus of cecropin A, and at the C-terminus of melittin within the chimeric protein, we then constructed an in-frame fusion *hcm1* gene using the following procedure. First, a sequence encoding the polylinker plus the active domains of cecropin A and melittin (with the flexible hinge between them) was PCR-amplified with the primer pair P3/P5 (Table 2) by using the *pep4* gene as the template (Fig. 1B). Next, this DNA fragment was ligated at the BamHI site to the DNA sequence that had been PCR-amplified with the primers Hpa1-F/Hpa1-R2 by using the *hpa1* gene as the template

(Fig. 1B). This construct (*hcm1*) was then ligated into the expression vector pET30a(+) at the NdeI and XhoI sites, producing the recombinant pHcm1 (Fig. 1B, Table 2). All the constructs were sequenced to verify correct reading frames.

Protein expression and purification

To express the chimeric proteins and Hpa1, which is used as the positive control for HR induction and the negative control for antimicrobial activity in this study, the constructs described above were transformed into host strain BL21 (DE3) (Table 1) by heat-transformation as described (Novagen pET System Manual; Novagen, USA), producing expression strains BLHcm1, BLHpa1, BLPep1, BLPep2, BLPep3 and BLPep4 (Table 1). Protein expression was performed as follows. A single colony of the expression strains was added into 200 ml LB containing Km at 25 μg ml^{-1}. After incubation with shaking at 200 r.p.m. at 37°C for 12 h, the 200 ml culture was added to 20 l of fresh LB containing Isopropyl β-D-thiogalactopyranoside (IPTG, Sigma) at 0.5 mM final concentration, and fermented in a NLF22 tank (Bioengineering AG, Switzerland) at 25°C for 16 h. After the cells were harvested by centrifugation, 1 g cell pellets were resuspended in 5 ml of PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4) containing 20% glycerol, 5 U ml^{-1} DNase I, and 5 μl of the protease inhibitor PMSF. The bacterial cells were lysed by sonication (20 kHz, 20 min). After centrifugation at 15 000 *g* for 15 min at 4°C, the supernatants were divided into two parts. One was incubated in a water bath at 100°C for 10 min and then re-centrifuged at 15 000 *g* for 10 min at room temperature. This supernatant, known as CFEP (Wei *et al*., 1992; Dong *et al*., 1999), was used directly for HR induction in tobacco and antimicrobial activity assays *in vitro*. In order to purify Hpa1 and the chimeric protein Hcm1, the second part of the supernatant was used as a source of membrane fragments, since the α-helices of Hpa1, cecropin A and melittin are all reported to bind to membranes of the targets (Hristova *et al*., 2001; Ferre *et al*., 2006; Glättli *et al*., 2006; Oh *et al*., 2007; Wang *et al*., 2007; Ji *et al*., 2010). After these supernatants was centrifuged at 200 000 *g* for 1 h at 4°C, 1 g cell membrane residues were resuspended in 5 ml of PBS buffer with 2% final concentration of dodecyl maltoside (DDM) detergent (Sigma). After shaking for 2 h at 4°C, the resuspensions were re-centrifuged at 200 000 *g* for 1 h at 4°C. The resulting supernatants were used to purify Hpa1 or Hcm1 proteins by a HisTrapTM^{FF} column following the GE Healthcare Purification Manual (GE Healthcare, Germany). The purified proteins were quantified using an Easy Protein Quantitative Kit (TransGen Biotech, Beijing, China) and a NANODROP 1000 Spectrophotometer (Thermo), and also analysed on a 12% SDS-PAGE gel (Bio-Rad, USA) and verified by Western immunoblotting.

HR induction in tobacco

The HR assay was performed as described by Zou and colleagues (2006). Hpa1 and the chimeric proteins at 0.1 μM, in the form of CFEPs extracted from the expression strains, were tested for the ability to elicit the HR on tobacco *N. tabacum* cv. Xanthi nn following infiltration into plant leaf tissues by needleless syringes. PBS buffer was used as the negative control. Plant responses were scored 24 h post inoculation. The plants used for the HR induction test were first transferred from the greenhouse into the laboratory one day prior to infiltration. Three leaves of each of three plants were used for each experiment, which was repeated three times with similar results.

RT-PCR assays for HR maker gene expression

To investigate whether HR marker-gene expression is activated by the fusion protein Hcm1, total RNA isolated from leaves of *N. tabacum* cv. Xanthi nn was used for RT-PCR. Eight hours following infiltration of the purified protein Hcm1 (10 μg ml^{-1}) into tobacco leaves, the RNA was extracted using Trizol (Invitrogen, USA), treated with DNase I (Takara, China) and purified. To confirm that there was no DNA contamination in the extracts, primers (Table 2) designed specifically to amplify the HR marker genes *HIN1, HSR203J* and *PR1-a* (Gopalan *et al*., 1996; Bowling *et al*., 1997; Pontier *et al*., 1998; Takahashi *et al*., 2004) were used to confirm that there were no PCR products generated using the extracts directly as templates (data not shown). Reverse transcription of 2 μg total RNA was carried out using an RNA PCR kit (AMV) with random primers (Order no. D3801 provided by the manufacturer, Takara, China). The reaction was performed at 30°C for 10 min, 42°C for 1 h, and then inactivated at 75°C for 5 min. One microlitre of the cDNA products was used as the template for PCR amplification of the HR marker genes with the specific primers (Table 2). Tobacco leaves infiltrated with purified Hpa1 protein or PBS buffer were used as the positive and negative controls respectively. Following an initial incubation at 95°C for 5 min, the PCR included 35 cycles of 95°C for 50 s, 53°C for 30 s, and 72°C for 40 s; the final incubation was at 72°C for 7 min. The RT-PCR products were subjected to electrophoresis in 1% agarose gels and were then sequenced to confirm that the primers used for amplifying the HR marker genes were specific.

Antimicrobial screening for the fusion proteins

To determine whether the chimeric proteins could inhibit the growth of plant pathogenic bacteria and fungi, we tested the CFEPs that were prepared from the expression strains producing Pep1, Pep2, Pep3, Pep4 and Hcm1 respectively. We spread 100 μl of *X. oryzae* pv. *oryzicola* strain RS105 at approximately at 1×10^6 cfu ml^{-1} onto fresh NA plates. Then, sterile filter paper discs (5 mm diameter) were placed on the surface of the NA. Each disc was dotted with 3 μl of the respective CFEPs, which had been sterilized through 0.22 μM-pore-size filters. Kanamycin at 10 μg ml^{-1} and PBS buffer were used as the positive and negative controls respectively. The plates were incubated at 28°C for 2 days and then antimicrobial haloes were recorded. Similar results were observed for three replicates.

MIC and ED_{50} of the antimicrobial protein Hcm1

For MIC assessment, the purified chimeric protein Hcm1 was solubilized in sterile Milli-Q water to a final concentration of 100 μM and then sterilized through a 0.22 μM filter. For MIC

assessment, dilutions of Hcm1 were made to obtain final concentrations of 75, 60, 50, 40, 35, 30, 25, 20, 15, 12.5, 10, 7.5, 5, 2.5, 1.25 and 0.625 μM. An aliquot (100 μl) of each dilution was mixed with 100 μl of the bacterial cells or the fungal spore suspensions (1×10^4 cfu ml^{-1}) to be used as indicators (Tables 1 and 3), and diluted to a total volume of 1 ml. After 12 h incubation for bacteria and 4 h for fungi, 20 μl of the bacterial mixtures was diluted and spread on growth medium plates, and 5 μl of the fungal mixtures was dripped onto microscope slides. Single bacterial colonies were counted using Quantity One software (Bio-Rad, USA) and germinated spores were counted under the microscope (OLYMPUS IX71, Germany). MIC testing was replicated twice for each microbial species. Positive controls used water instead of the fusion protein Hcm1, and the negative controls used Hcm1 without bacteria or fungi.

Inhibition of growth (I) was calculated as a percentage of the positive control using the following equation: $I = 100 \times [(C - T)/C]$, where C is the cfu ml^{-1} of the control, and T is the cfu ml^{-1} of the treatment. The MIC was defined as the lowest protein concentration that allows less than 1% growth of the tested microbe, and the ED_{50} was that protein concentration that causes 50% death (Montesano *et al.*, 2003).

Antimicrobial spectrum for Hcm1

To investigate the antimicrobial spectrum for Hcm1, the following were used: *E. coli* BL21 (DE3), *R. solanacearum* ZJ3721, *X. oryzae* pv. *oryzicola* RS105 and *P. syringe* pv. *tomato* DC3000 as Gram-negative bacteria; *B. subtilis* B168 as a Gram-positive bacterium; and *F. graminearum* ZF21, *M. oryzae* Guy11, *A. alternata* TBA28 and *T. cucumeris* JS01 as agronomically important pathogenic fungi (Table 1). To test for inhibition of bacteria, 100 μl of the above bacterial suspensions at 1×10^6 cfu ml^{-1} was spread on fresh growth plates and sterilized filter paper discs soaked in Hcm1 at 1 μM and 5 μM were placed on the surface of the plates. To test for inhibition of fungi, 5-mm-dia mycelial discs were placed in the centre of PDA plates and then 5-mm-dia holes were made around the mycelial discs using a hole puncher. Ten microlitres of Hcm1 solutions at 1 μM and 5 μM was added into the holes on the PDA plate respectively. The plates were incubated at 28°C for 3–5 days depending on different fungal growth rates. The inhibition haloes of Hcm1 against bacteria and fungi were recorded. Kanamycin (10 μg ml^{-1}) and carbendazim (50 μg ml^{-1}) were used as the positive controls for bacteria and fungi, respectively, while Hpa1 (5 μM) was used for the negative control.

Inhibition of fungal spore germination by Hcm1

To investigate whether Hcm1 affects fungal spore germination, *M. oryzae* Guy11 and *F. graminearum* ZF21 (Table 1) were used as the targets. Hcm1 solution at 5 μM was mixed with the fungal spores (1×10^3 spore μl^{-1}) and incubated for 0, 4 and 8 h at 25°C. Spore germination was assessed under bright-field microscopy (OLYMPUS IX71, Germany). Hpa1 solution at 5 μM was used as the negative control. Similar results were obtained from two replicates.

Kinetics of survival of plant pathogenic bacteria treated with Hcm1

The effect of Hcm1 on bacterial survival *in vitro* was determined for three plant pathogenic bacteria. Cultures at 4×10^6 cfu ml^{-1} of *X. oryaze* pv. *oryzicola* RS105 and *R. solanacearum* ZJ3721 in NB, or of *P. syringe* pv. *tomato* DC3000 in Kings B broth, were incubated with the AMP Hcm1 at 2 μM, or with Hpa1 at 2 μM as the negative control. Aliquots of 100 μl were removed at 30 min intervals during the 3 h incubation and diluted 10-fold before plating on the corresponding growth media. Colonies were counted after 48 h incubation at 28°C, and the percent survival was determined in relation to the starting cultures.

Susceptibility of Hcm1 to protease degradation

Digestion of Hcm1 and Hpa1 by protease K (Sigma, USA) was tested by treating 10 μM protein with 1 U protease K in 90 μl of 100 mM Tris Buffer (pH 7.6) at room temperature. Protein cleavage after 5, 10, 15, 30, 45, 60 and 75 min was monitored by an Easy Protein Quantitative Kit (TransGen Biotech, Beijing, China) using a Thermo NANODROP 1000 Spectrophotometer. Digestion was calculated as a percentage of the original protein concentration using the following equation: $D = 100 \times (1 - T/T_0)$, where T is the protein concentration with protease K at the above time points, and T_0 is the original protein concentration.

Evaluation of increased resistance or reduced disease severity by Hcm1 treatment of plants before inoculation with plant pathogens

For testing the effect of prior application of Hcm1 on reducing plant pathogen infections on different host plants, the following pathogens that cause important crop diseases were chosen. We looked at infection by TMV of *N. tabacum* cv. Xanthi nn, which induces HR-like necrotic lesions to TMV infection (Ehrenfeld *et al.*, 2008); at the incidence of rice blast-associated lesions caused by *M. oryzae* on *O. sativa* cv. CO-39; and at the incidence of tomato wilt caused by *R. solanacearum* on *Solanum lycopersicum* cv. Suhong 2003. Plants at appropriate ages were sprayed twice at a three-day interval by either Hcm1 (1.5 μM plus 0.5% Tween 20), Hpa1 (1.5 μM plus 0.5% Tween 20), or PBS buffer, and then inoculated by the respective pathogens 3 days after the second spraying.

A crude inoculum of TMV was freshly prepared by homogenizing infected tobacco leaves (1 ml deionized, distilled water per 1 g diseased leaf), followed by filtering through gauze, diluting 1:100 with sterilized water, and then mixing with emery powder. Three days following treatment with Hcm1, the crude inoculum was rubbed gently onto the upper surface of tobacco leaves of 2-month-old plants using cotton tips. After 3 days, the resulting necrotic lesions were counted and the necrotic area per leaf area was calculated using the software Quantity One v4.62. TMV disease severity was rated as follows: 0, below 1% necrotic area/leaf area; 1, 1–10% necrotic area; 2, 10–25% necrotic area; 3, 25–40% necrotic area; 4, over 40% necrotic area/leaf area (Enyedi *et al.*, 1992).

For rice blast, a fresh spore suspension of *M. oryzae* Guy11 was prepared from 14-day-old cultures and diluted to the concentration of 1×10^5 spore ml^{-1} in sterilized water containing 0.2% (w/v) gelatin. The suspension was sprayed onto 4-week-old susceptible rice, *O. sativa* cv. CO-39, which had been previously sprayed with Hcm1, Hpa1 or PBS. Inoculated plants were placed in the dark in a moist chamber at 28°C for the first 24 h, and were then transferred to another moist chamber with a photoperiod of 12 h under fluorescent lights (Zhang *et al*., 2009a). Five days after inoculation, diseased rice blades were photographed and the percentage of diseased leaf area was recorded and calculated as described by Fang and Dean (2000). The disease index of individual leaves was calculated based on the following severity: 0, below 1% spotted area per leaf area; 1, 1–10% spotted area; 2, 10–25% spotted area; 3, 25–40% spotted area; 4, over 40% spotted area.

For tomato bacterial wilt, a fresh suspension of *R. solanacearum* ZJ3721 was adjusted to 1×10^8 cfu ml^{-1} and injected by needled syringes into the stems of 1-month-old tomato seedlings that had been previously sprayed with Hcm1, Hpa1 or PBS. The inoculated plants were kept in the greenhouse for 16 days. At 8 days post inoculation (dpi) and 16 dpi, diseased plants were photographed and the bacterial wilt rate was calculated. The wilt severity per plant was calculated as follows: 0, below 25% wilted leaves; 1, 25–55% wilted leaves; 2, 55–85% wilted leaves; 3, over 85% wilted leaves; 4, entire plant wilted (Li *et al*., 2010).

All together, each sample was tested on 10 individual plants and the experiments were repeated three times. The disease index was calculated by the following equation: $DI=100 \times \Sigma(n \times I)/(N \times L)$, where DI is disease index, I is the severity level, n is the number of diseased plants for each severity level, N is the total number of treated plants, L is the highest severity level seen in this investigation. Inhibition rate (IR) was estimated by the equation: $IR=100 \times (CD - TD)/CD$, where CD is the disease index of the PBS-treated plants and TD is the disease index of Hcm1- or Hpa1-treated plants. All of the statistical analyses were done using the software SSPS v13.0.

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