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. *oryzaica*, 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 (ED50) 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 (Allano and Collmer, 1996; Ferre et al., 2006; Cavallarin et al., 1998; Lehrer et al., 1998; Hancock, 2001; Shai, 2002; Ferre et al., 2005; Glättli et al., 2006; Raghuraman and Chattopadhyay, 2007). An α-helix in melittin, AVLKVTTLGL, 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 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, AVLKVTTLGL, 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 Hpa1Xoo from X. oryzae pv. oryzicola, the causal agent of rice bacterial blight, is attributed to two α-helices involved in coiled-coil protein interactions, SEKQLDQLCLQIALLQ and PFTQMLM HIVGEILQAA, 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 Hpa1Xoo (Zou et al., 2006), predicted two α-helices, ISEKQLDQLLCQLISALLQ and PFTQMLM HIVGEILQAA, at the N- and C-termini of the protein.
C-termini (Fig. 1). Since mutations in the N-terminal α-helices of Hpa1Xoo 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; Giätti 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, KLFFKIEKV, of cecropin A, plus a stop codon; pep2, where the pep1 gene was linked directly at its 3’ end to the sequence encoding the α-helix, AVLKVLTTGL, of melittin; pep3, where 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.

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,
individual cell-free elicitor preparations (CFEPs) of Hpa1, Pep1, Pep2, Pep3 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. oryzae 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.

| Strains or plasmids | Properties | Source |
|---------------------|------------|--------|
| Strains E. coli     |            |        |
| DH5x                | F-, F80dlacZαM15(ΔlacZYA-argF)U169 deoR recA endA1 | Invitrogen |
| BL21(DE3)           | F-, ompT hsdSB (R' mB1 gal dcm) (DE3) | Novagen |
| BLHcm1              | Transformant of BL21(DE3) with pHcm1, Km' | This study |
| BLPep1              | Transformant of BL21(DE3) with pPep1, Km' | This study |
| BLPep2              | Transformant of BL21(DE3) with pPep2, Km' | This study |
| BLPep3              | Transformant of BL21(DE3) with pPep3, Km' | This study |
| BLPep4              | Transformant of BL21(DE3) with pPep4, Km' | This study |
| BLHpa1              | Transformant of BL21(DE3) with pHpa1, Km' | This study |
| B. subtilis         | trpC2      | This lab |
| X. oryzae pv. oryzae|            |        |
| RS105               |            | 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' | 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, Apβ | TaKaRa |
| pET30a (+)          | pBR322 origin, F1 origin, lacI, His-Tag, S-Tag, Km' | Novagen |
| pHcm1               | hcm1 in pET30a(-) at Ndel and Xhol sites, His-tagged, Km' | This study |
| pPep1               | pep1 in pET30a(-) at Ndel and Xhol sites, His-tagged, Km' | This study |
| pPep2               | pep2 in pET30a(-) at Ndel and Xhol sites, His-tagged, Km' | This study |
| pPep3               | pep3 in pET30a(-) at Ndel and Xhol sites, His-tagged, Km' | This study |
| pPep4               | pep4 in pET30a(-) at Ndel and Xhol sites, His-tagged, Km' | This study |
| pHpa1               | hpa1 in pET30a(-) at Ndel and Xhol sites, His-tagged, Km' | This study |

a. Apβ = ampicillin resistance; Km' = kanamycin resistance; Rif' = rifampicin resistance.

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...
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 transcription 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).

**Fig. 2. In vitro assays of chimeric proteins for antimicrobial activity against X. oryzae pv. oryzae 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. oryzae 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. oryzae RS105 at approximately 1 × 10⁸ 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 Tag 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.

**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 BL Pep1, BL Pep2, BL Pep3 and BL Pep4 (data not shown). This suggested that Hcm1 may be toxic to E. coli, possibly through binding to the bacterial surface, 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 HisTrapTMFF 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.
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 μgm l⁻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 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.

| Primers | 5′- to 3′-sequence, restriction sites underlined | Description |
|---------|-------------------------------------------------|-------------|
| Hpa1-F  | AACATATGTAAGAAGTCCTTGTAAGAC | 414 bp hpa1 gene |
| Hpa1-R1 | TTTCGAGTTTACGCTATGCTTCGCTG | XhoI site was added at 3′-termi of hpa1 |
| Hpa1-R2 | TTGGACCTTACGCTATGCTTCGCTG | BamHI site was added at 3′-termi of hpa1 |
| P1      | CTGGAATCTACATTAAAAAGTTTCCTGAGCAGTCACT | Sequence for KLKKIEKV was fused to C-terminus of Hpa1 |
| P2      | AACAGTCAAGCAGACCTGTAAGCCTAAGAC | Sequence for AVLKVLTTLG was fused to C-terminus of Hpa1 |
| P3      | CAGGATCCGGCCGGCGGTTCCGCGGTTAAGTGGTGGAAACTCTTTAAG | Sequence for the polylinker DPGGFGGKW was fused before the α-helix of cecropin |
| P4      | TTGGACCTTTTAAACTTCCTTTAAGAG | Sequence for the flexible hinge QGQGIG was fused after the α-helix of cecropin |
| P5      | AACAGTCAAGCAGACCTGTAAGCCTAAGAC | Sequence for the α-helix of melittin was fused after the flexible hinge |
| Hin1-F  | GAACGGAGCCATTATGGCCCTTCC | 867 bp HIN1 gene |
| Hin1-R  | CATGTATATCAATGAACACTAAACGCCGG | 618 bp HSR203J gene |
| HSR203J-F | TTGAACACCAACATTCCGCCG | 495 bp PR1-a gene |
| PR1-a-R | GCCGTTCCTTCACACCATTGGCCTTCA | 618 bp HSR203J gene |
| EF1a-F  | AGACCACAAAGTACTACTGAC | 495 bp EF1a gene |
| EF1a-R  | CCACCATCTTTGTACATCC | 495 bp EF1a gene |

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.

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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).
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).

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

| 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 |

Fig. 5. Inhibition of fungal spore germination by the chimeric protein Hcm1. Fresh spore suspensions (1 × 10³ 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.
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 mM), 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...
spindle spots), and the severity of plant wilting was assessed for tobacco 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 $\alpha$-helices of cecropin A and melittin, are necessary for these two activities (Fig. 1). The polylinker may stabilize the two $\alpha$-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 $\alpha$-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.

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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 (%) | DI | IR (%) |
|-----------------------------|-----------|----------------|----------------------------|---------------|----|--------|
| Tobacco mosaic              | Hcm1      | 98.5 ± 19.49** | 24.1 ± 6.61*               | ND            | 55.73* | 46.43  |
|                             | Hpa1      | 151.6 ± 30.98**| 28.5 ± 6.97**              | ND            | 61.42** | 35.71  |
|                             | PBS       | 258.4 ± 63.15**| 45.3 ± 7.71**              | ND            | 93.33** | –      |
| Rice blast                  | Hcm1      | 48.5 ± 11.37*  | 13.3 ± 3.38*               | ND            | 48.32*  | 47.28  |
|                             | Hpa1      | 95.6 ± 13.24*  | 38.2 ± 6.94*               | ND            | 84.02*  | 5.53   |
|                             | PBS       | 105.3 ± 15.68* | 41.4 ± 3.57**              | ND            | 91.22*  | –      |
| Tomato bacteria wilt        | Hcm1      | ND             | ND                         | 74.8 ± 8.52*  | 55.13*  | 38.89  |
|                             | Hpa1      | ND             | 92.7 ± 5.94*               | ND            | 83.32*  | 7.41   |
|                             | PBS       | ND             | 96.7 ± 4.87*               | ND            | 90.27*  | –      |

*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.

Wilt rate equals percentage of leaves wilted per plant (see Experimental procedures).

DI means disease index (see Experimental procedures).

IR presents inhibition rate (see Experimental procedures).
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 compartimentalization 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

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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 intrinsically to plant tissues will degrade Hcm1. Engineered sequence changes to cloned AMPs have been shown to enhance desirable properties (Andreu et al., 1983; Cavallarín 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. oryzae* strain 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% K2HPO4, 1.5% MgSO4.7H2O, 1.5% agar, pH 7.2) at 30°C. All of the plant pathogenic fungi, including *F. graminearum* TBA28 and *T. cucumeris* RS105, *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 mg 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 tobasanovirus TMV infects) (Ehrenfeld et al., 2008), *O. sativa* cv. CO-39 and *S. lycopersicum* cv. Suhoong 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. oryzae* 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, AVLKKVLTTGL, 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 Xhol 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 BamHII 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 BamHII 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 BamHII 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 Xhol 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 GQQG (van Noort et al., 2004) was added between these two domains with the primer pairs Hpa1-F/P4 and Hpa1-F/P5 (Table 1, Fig. 1B), respectively, by using the *pep7* gene as the template. The final PCR product was ligated into pET30a (+) at the NdeI and Xhol sites, producing the constructs 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 BamHII 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
Hcm1 induces HR and inhibits plant pathogens

(1 h at 4°C). This construct (hcm1) was then ligated into the expression vector pET30α+(+) 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 2 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 Na₂HPO₄ and 2 mM KH₂PO₄, 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; Glätti et al., 2006; Oh et al., 2007; Wang et al., 2007; Ji et al., 2010). After these supernatants were 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 HitTrapTM 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 × 10⁶ 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 ED₅₀ 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 × 10⁵ 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: \( I = 100\times \left( \frac{C - T}{C} \right) \), 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 ED₅₀ 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 × 10⁶ 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 added 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 × 10⁵ 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 × 10⁶ cfu ml⁻¹ of *X. oryzae* 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: \( D = 100\times \left( 1 - \frac{T}{T_0} \right) \), 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., 1992); 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. Suohon. 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 v4.6.2. 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* Gyu11 was prepared from 14-day-old cultures and diluted to the concentration of 1 × 10^6 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, 25–85% spotted area; 4, over 85% spotted area.

For tomato bacterial wilt, a fresh suspension of *R. solanacearum* ZJ3721 was adjusted to 1 × 10^6 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 × Σ(n × l)/N × l, where DI is disease index, l 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 × (CD – TD)/CD, where CD is the disease index of the PBS-treated plants and TD is the disease index of Hcm-1- or Hpa1-treated plants. All of the statistical analyses were done using the software ssps v13.0.

Acknowledgements

We are grateful to Dr Alan Collmer of Cornell University for his critical suggestions and helpful discussions on the experiments in this study, and to Dr Candace W. Collmer at Wells College for her kindly reading and re-editing the manuscript. This work was supported by the State Key Basic Research and Development Project of China (2011CB16141), the Natural Science Foundation of China (30710139092, 31071656), the National Transgenic Major Program (2008ZX08001-002) and the Special Fund for Agro-scientific Research in the Public Interest (NYHYZX07-056, 201003067-09).

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