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Synthetic Ultrashort Cationic Lipopeptides Induce Systemic Plant Defense Responses against Bacterial and Fungal Pathogens[▽]

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A new family of synthetic, membrane-active, ultrashort lipopeptides composed of only four amino acids linked to fatty acids was tested for the ability to induce systemic resistance and defense responses in plants. We found that two peptides wherein the third residue is a D-enantiomer (italic), C_{16}-KK*K*K and C_{16}-KL*L*K, can induce medium alkalinization of tobacco suspension-cultured cells and expression of defense-related genes in cucumber and *Arabidopsis* seedlings. Moreover, these compounds can prime systemic induction of antimicrobial compounds in cucumber leaves similarly to the plant-beneficial fungus *Trichoderma asperellum* T203 and provide systemic protection against the phytopathogens *Botrytis cinerea* B05, *Pseudomonas syringae* pv. *lachrimans*, and *P. syringae* pv. *tomato* DC3000. Thus, short cationic lipopeptides are a new category of compounds with potentially high utility in the induction of systemic resistance in plants.

Antimicrobial peptides (AMPs) (12 to 50 amino acids long), known also as innate immunity host defense peptides or innate defense regulators, are key components of the innate immune system in all phyla, providing a fast-acting defense against invading pathogens (5, 16, 25). The amino acid residues most abundant in AMPs are hydrophobic and cationic, which results in electrostatic attraction to negatively charged microbial envelopes, leading in several cases to lysis of the cell membrane (35). This can explain their specificity to microbes and their lower toxicity to plant and animal cells lacking charged membrane phospholipids (23). However, the rationale that peptide activity is a direct consequence of membrane-disrupting capabilities is a generalization that might not account for all forms of antimicrobial action (5, 6, 25, 30). In humans and other mammals, these peptides have both direct and indirect antimicrobial activities, such as the abilities to promote wound healing and to modulate adaptive immunity (4).

A subfamily of AMPs with strong antimicrobial activity includes lipopeptides, which are produced nonribosomally in bacteria and fungi. Lipopeptides consist of a short linear or cyclic peptide sequence with a net positive or negative charge to which a fatty acid moiety is covalently attached at the N terminus (40).

Plant diseases play a significant role in the devastation of natural resources in agriculture. Growing awareness of the environmental damage caused by the use of chemical substances against plant diseases has raised the need to study biological alternatives (28, 29). Because AMPs are one of the most ancient and widespread defense strategies in nature and practically do not induce bacterial resistance, they are of interest for potential applications in medicine and agriculture (7).

Plant induced systemic resistance (ISR) is a phenomenon whereby resistance to a wide range of pathogens, including fungi, bacteria, and viruses, is systemically induced by beneficial microorganisms or treatment with microbial components or by a diverse group of structurally unrelated organic and inorganic compounds (18). An important aspect of ISR is the priming effect, meaning that defense responses are not activated directly but are accelerated upon pathogen or insect attack, resulting in enhanced resistance to the attacker encountered. ISR does not usually require substantial transcriptional reprogramming in the host plant before a pathogen attack, thus affording significantly higher levels of fitness (43). Cyclic lipopeptides, such as surfactins and fengycins from the bacterial plant biocontrol agent *Bacillus subtilis* or massetolide A from *Pseudomonas fluorescens* SS101, have been shown to stimulate the induction of ISR in bean and tomato plants (31, 32, 41). Nevertheless, certain AMPs of microbial origin have significant phytotoxicity that limits their direct use as plant protection products (28). Furthermore, the high cost of long peptides and lipopeptides limits their utilization in agriculture. Searches for shorter, more potent, nontoxic, and more stable peptides have led to the identification of synthetic peptides with broader and higher activity than their natural counterparts (14, 27, 29, 45). We recently reported a new family of synthetic ultrashort lipopeptides with a broad spectrum of in vivo and in vitro antimicrobial activity against human-pathogenic yeasts, fungi, and bacteria (22, 42), also affecting phytopathogenic fungi and bacteria (23), with little or no phytotoxicity. Studies of their possible modes of action support a membranolytic or detergent-like effect, probably via the carpet mechanism (26, 37, 47). This mode of damage should make it difficult for the microorganisms to develop resistance. These lipopeptides are composed of only four L- and D-amino acids linked to fatty acids. The sequence of the peptidic moiety and the length of the fatty acyl group determine the specificities of

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TABLE 1. Primers used for quantitative PCR expression analysis in *Arabidopsis*

Gene (AGI code)[a]	Primer sequence	
	Forward	Reverse
UBQ10 (at4g05320)	GGCCTTGTATAATCCCTGATGAATAAG	GGCCTTGTATAATCCCTGATGAATAAG
PR1 (at2g14610)	GAATTTTACTGGCTATTCTCGATTT	CTTCTCGTTCACATAATTCCCACG
PR2 (at3g57260)	ATGCTAGGCGATACCTTGCCA	CCGCATTCGCTGGATGTTT
PR5 (at1g75040)	TGGCGGCAAAGATTTCTACG	TTTGCAATCTCCCGATCCTC
vsp2 (at5g24770)	TGGGAACGTAGCCGAACTCTTA	CCCGAGCTCTATGATGTTTTGG

[a] AGI, *Arabidopsis* Genome Initiative.

the lipopeptides against bacteria, fungi, and mammalian cells (22). In the present work, we evaluated the ability of these compounds to act as inducers of systemic defense responses in plants and found two with high efficacy. Elucidation of their mode of action and interaction with plants and microbes will assist the improvement of peptide design with a view to targeting specific problems in agriculture and providing new tools for plant protection.

MATERIALS AND METHODS

Materials. Rink amide MBHA resin, 4-methylbenzhydrylamine resin, and 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were obtained from Calbiochem-Novabiochem AG (Switzerland). Aliphatic acids were purchased from Sigma Chemical Co. (Israel). Other reagents used for peptide synthesis included trifluoroacetic acid (Sigma), piperidine (Merck), *N*,*N*-diisopropylethylamine (Sigma), *N*-methylmorpholine (Fluka), *N*-hydroxybenzotriazole hydrate (Aldrich), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, and dimethylformamide (peptide synthesis grade; Biolab).

Peptide synthesis, acylation, and purification. Peptides were synthesized by a solid-phase Fmoc method on rink amide MBHA resin with an ABI 433A automatic peptide synthesizer. The lipophilic acid was attached to the N terminus of a resin-bound peptide by standard Fmoc chemistry, followed by peptide cleavage from the resin and purification by reverse-phase high-performance liquid chromatography (>98%) (2). The composition of the lipopolyamine was confirmed by electrospray mass spectroscopy and amino acid analysis.

***Cucumis sativus* seedling growth conditions and peptide application.** *C. sativus* seeds (Kfir; Gedera Seeds, Israel) were surface disinfested in 2% NaOCl for 2 min, thoroughly washed with sterile distilled water, and germinated under sterile conditions (48). After 6 days, seedlings with fully expanded cotyledons were transferred to small vials containing 3.1 nmol of P1, P2, or P3 diluted in water or of a mock solution. The vials were then placed in a sterile polycarbonate culture box (9). After 48 h, the seedlings were inoculated with 10 μl of a bacterial suspension as described in reference 49. Water was added after the first 24 h to the small vials as needed. Cotyledons were sampled at different times for RNA extraction, bacterial counting, or phenolic extraction.

Alkalinization assay. Tobacco suspension cells (BY2) were maintained in Murashige and Skoog medium (Sigma, St. Louis, MO) adjusted to pH 5.8 with KOH. For routine maintenance, 1 ml of a 1-week-old culture was transferred into 15 ml of medium in 150-ml flasks and maintained on an orbital shaker at 100 rpm in the dark at room temperature. A 1-ml aliquot of a cell suspension was transferred into each well of 12-well tissue culture plates (Corning, Corning, NY) and allowed to equilibrate on an orbital shaker at 120 rpm for 50 min. Peptides (3.1 to 6.2 nmol) were added to the cells, and the change in the pH of the medium was measured every 5 min for up to 25 min.

Phenolic extraction from cucumber cotyledons. Phenolic extraction was performed 48 h after inoculation with *P. syringae* pv. *lachrimans* according to reference 49. Briefly, fresh foliar material was ground to a fine powder in liquid N_2 and extracted in 80% acidified methanol (10 g [fresh weight]/100 ml). The mixture was maintained for 24 h in the dark under nitrogen. The extract was filtered through glass fiber and concentrated with a rotoevaporator. The aqueous residue was partitioned against hexane and ethyl acetate and subjected to acid hydrolysis. The hydrolysate was cooled and partitioned against ethyl acetate. This fraction was dried and resuspended in absolute methanol (2.5 g [fresh weight]/ml).

Microbial bioassay. Cells of *Pseudomonas syringae* pv. *lachrymans* were cultured in Trypticase soy broth. Crude phenolic extracts were further concentrated with a Speed-Vac and adjusted to 100 μl with absolute methanol. Different amounts of the concentrated samples were pipetted onto Trypticase soy agar plates and dried. Bacterial suspensions (200 μl) were mixed into 3 ml of soft Trypticase soy agar and overlaid on the dried plates. Antimicrobial activity of the extract was assayed 24 h after bacterial application and appeared as clear lytic circles on the plates. The inhibition rate was correlated to the diameter (in millimeters) of the lytic circle (49).

RNA extraction and gene expression analysis by quantitative reverse transcription (RT)-PCR. Total RNA was extracted from plant leaves and roots according to reference 49. RNA was DNase treated and further cleaned by using RNeasy mini columns (Qiagen). Total RNA (2 μg) was subjected to first-strand synthesis with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's procedure by using oligo(dT) as a primer. As a negative control, the same reactions were performed in the absence of the enzyme.

Primers for quantitative RT-PCR experiments with cucumber genes were as in reference 49 for the *hpl* and *pal1* genes and as in reference 38 for *prx*. *C. sativus* actin was used as the endogenous gene reference (forward, CGTGCTGGATT CTGGTGATGG; reverse, CGTGCTGGATTCTGGTGATGG).

Primers for *Arabidopsis* are shown in Table 1. The ubiquitin gene (*UBQ10*) was used as an endogenous gene reference in this case. PCR was carried out with a 20-μl reaction mixture containing SYBR green PCR Master Mix (PE Applied Biosystems), 500 nM primer (each forward and reverse primer), and 1/25 of the RT reaction mixture. Quantitative analysis was performed with the GeneAmp7300 sequence detection system (PE Applied Biosystems) under PCR conditions of 95°C for 15 s and 60°C for 1 min for 40 cycles. The absence of primer-dimer formation was examined in no-template controls. Six independent biological replicates were used in each analysis. Each sample was examined in triplicate by relative quantification analysis.

***Arabidopsis* seedling growth and bacterial inoculation assay.** For aseptic growth of seedlings, *Arabidopsis* ecotype Col-0 seeds were sterilized by treatment for 5 min in 70% ethanol, followed by 5 min in 50% bleach (2.63% sodium hypochlorite solution). They were then extensively washed with sterile water (five or six times), plated on Gamborg B5 medium with vitamins (Duchefa Biochemie, The Netherlands), and kept in darkness for 2 days and in light for other 5 days (22°C with a 16-h photoperiod at a light intensity of 100 $\mu E\ m^{-2}\ s^{-1}$ for 10 days). Fifteen seeds were dispensed into each well of a 12-well tissue culture plate with 1 ml B5 medium supplemented with 0.5% sucrose and 0.5 g/liter 2-(*N* -morpholino)ethanesulfonic acid (MES), pH 5.7. Seedlings were treated with 1.6 nmol of the different peptides. *P. syringae* pv. *tomato* DC3000 was grown in King's B medium (10 mg/ml protease peptone, 2 mg/ml K_2HPO_4, 10 mg/ml glycerol, 6 mM $MgSO_4$, pH 7.0) plus antibiotic at 28°C to log phase (optical density at 600 nm of 0.6 to 0.8) and then harvested by centrifugation for 30 s, followed by three washes with sterilized water. Bacteria were resuspended in sterile deionized water to an optical density at 600 nm of 0.02. Ten microliters (10^4 CFU/ml) of this bacterial suspension was added to each well. Prior to quantification, the seedlings were washed in sterile water, then in 70% ethanol, and finally in sterile water again. The tissue was then weighed and ground in 10 mM $MgSO_4$. Serial dilutions were plated on King's B plates containing appropriate antibiotics (10).

***Botrytis cinerea* infection of cucumber and *Arabidopsis* leaves.** Cucumber seedlings (Kfir variety; Zraim Gedera, Israel) were grown in soil in 250-ml pots in a controlled environment. One hundred microliters of a 12.5 μM water-diluted solution of peptides P1 and P2 or a mock solution (water) was allowed to infiltrate the second and third leaves of 10-day-old plants by using a syringe without a needle. Younger leaves were inoculated 24 h later with 5-mm-diameter mycelial agar discs of *B. cinerea* strain B05 taken from 7- to 10-day-old cultures maintained on potato dextrose agar. The disc ($\sim 2 \times 10^6$ spores) was placed in the middle of each leaflet (11). After 24 h, the discs were removed and disease development was assessed 3 to 4 days after inoculation. Two rosette leaves of 5-week-old *Arabidopsis thaliana* ecotype Colombia plants grown under a 10-h

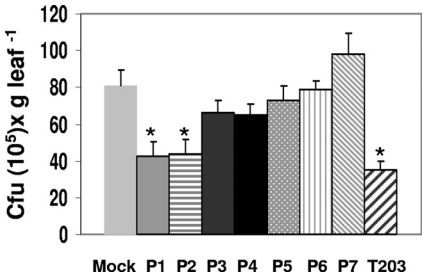

FIG. 1. Effect of ultrashort peptide application at the root site on multiplication of *P. syringae* pv. *lachrimans* in challenged cotyledons. Cucumber seedlings were treated with 3.1 nmol of different peptides (P1 to P7) or with water (Mock) 48 h prior to bacterial challenge. CFU counts of surviving bacteria were assayed 48 h after inoculation. Seedling inoculation with the beneficial fungus *T. asperellum* T203 was used as a control for the induction of systemic protection (42, 48). The results shown are averages (± standard errors) of three independent experiments. *, significantly different from the control (one-way factorial ANOVA; $P \ll 0.001$).

photoperiod at 21°C were infiltrated with peptide or mock solution as described above. Neighboring leaves were inoculated with 2,500 B05 spores in a 3-μl drop of diluted sterile grape juice (Carmel Tirosh, Israel). Disease development was assessed 3 to 4 days after inoculation.

Statistical analysis. All statistical data analyses were performed with the statistical software package R (http://www.R-project.org). Three- and one-way factorial analyses of variance (ANOVA) were performed with the ANOVA function in R on linear fitted models. Post-hoc tests were performed usually as Tukey honest significant differences (TukeyHSD or T method) (39). The normality of group data was tested by the Cramer test (3) with random deviates of a normal distribution with the respective group mean and group standard deviation in R. The log likelihood ratio test (G test) of independence with Williams' correction was performed as described by Sokal and Rohlf (39). Values of $P < 0.05$ were considered significant.

RESULTS

Screening of AMPs for induction of systemic resistance in cucumber seedlings. Seven peptides designated P1 (C_{16}-KK*K*K), P2 (C_{16}-KL*L*K), P3 (C_{16}-KA*A*K), P4 (C_{16}-KGG*K*), P5 (C_{14}-KL*L*K) (22), P6 (C_{12}-*L*6K6) (24), and P7 (AMP 1D) (34) (amino acids in italics are the D-enantiomers) were assayed for induction of systemic resistance to the foliar pathogenic bacterium *P. syringae* pv. *lachrimans* in cucumber seedlings when applied at the root site.

One-way factorial ANOVA ($P \ll 0.001$) revealed that at 3.1 nmol, only peptides P1 and P2 could significantly ($P \ll 0.001$) induce systemic protection comparable to that afforded by spores (10^5 ml^{-1}) of *Trichoderma asperellum* T203, a well-known fungal inducer of plant systemic responses and of antimicrobial leaf compounds in cucumber plants (49) (Fig. 1). The two peptides were chosen for further characterization together with another peptide (P3) which did not show statistically significant systemic protection ($P = 0.528$).

Alkalinization assay of tobacco cell suspension medium. Alkalinization activity was monitored by measuring the culture medium pH every 5 min after 3.1 or 6.25 nmol (equivalent to 2 or 4 μg, respectively) of elicitor was added (Fig. 2A and B). At both concentrations, peptide P3 barely induced a change of 0.15 pH unit, which is not significantly higher than the control treatment. At the higher concentration, peptides P1 and P2 both induced increases of approximately 0.6 to 0.5 pH unit over a 20-min period (Fig. 2B). A lower peptide dose (effective in inducing ISR in cucumber plants, Fig. 1) induced less medium alkalinization (0.5 to 0.4 pH unit) but still significantly more ($P < 0.001$) than the control in the first 10 min (Fig. 2A) of the assay.

Induction of defense-related gene expression and antimicrobial compounds in cucumber plants. Induction of three defense response-related genes, i.e., those for hydroxyperoxide lyase (*hpl*), phenylalanine ammonia lyase (*pal1*), and peroxidase (*prx*), was monitored by quantitative PCR 24 h after a bacterial challenge in cotyledons of cucumber seedlings pretreated at the root site for 48 h with 3.1 nmol of the different peptides. Gene expression was compared to that of seedlings challenged with the pathogen only, treated only with the peptide, or left untreated. Peptide (P1 and P2) pretreatment prior to a bacterial challenge significantly potentiated gene expression in comparison to that of the controls (Fig. 3A), as confirmed by one-way factorial ANOVA, followed by TukeyHSD with $P < 0.05$. P3, which cannot afford protection (Fig. 1), did not induce significant upregulation of the genes.

Aglyconic phenol fractions were extracted from plants after

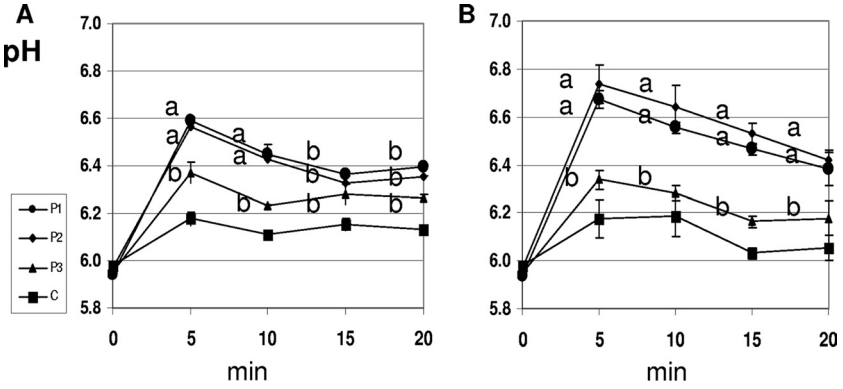

FIG. 2. Medium alkalinization by tobacco BY2 suspension cells upon addition of ultrashort lipopeptides. A 3.1-nmol (A) or a 6.25-nmol (B) sample of lipopeptide P1, P2, or P3 or 5 μl of water (control [C]) was added to 1 ml of cells, and the pH was measured every 5 min. The results shown are averages (± standard deviations) of three independent experiments with six repeats for each time point. A three-way factorial ANOVA of time, concentration, and peptide revealed significance for all three factors. a, significantly different from the control. b, not significantly different from the control.

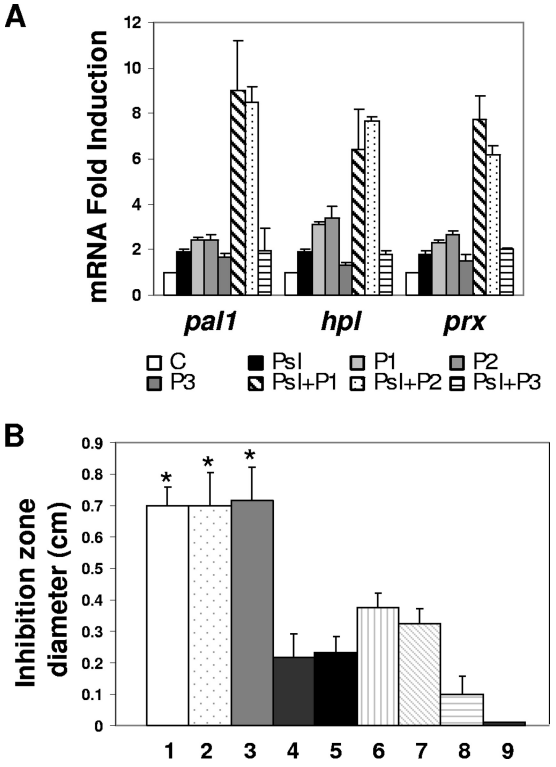

FIG. 3. Systemic induction of plant defense responses by synthetic peptides P1, P2, and P3 in cucumber plants. (A) Induction of defense-related gene expression. A 3.1-nmol sample of each peptide was applied at the root site of cucumber seedlings for 48 h. Total RNA was extracted 24 h after a bacterial challenge from a pool of three leaves and used for quantitative RT-PCR analysis of *hpl*, *pal1*, and *prx* gene expression. *n*-fold mRNA induction was compared to that in untreated seedlings (control [C]), to that in seedlings infected with the bacteria without previous peptide treatment (*P. syringae* pv. *lachrimans* [Psl]), and to that in seedlings treated with peptides (P1, P2, and P3) but not infected with bacteria. The results shown are averages of six independent treatment replicates ± the standard error tested by one-way factorial ANOVA, followed by TukeyHSD with $P < 0.05$ in comparison to the controls. (B) Growth-inhibitory activity toward *P. syringae* pv. *lachrimans* bacteria of phenol aglyconic extracts from plants pretreated with *T. asperellum* T203 or ultrashort synthetic peptides. A bioassay compared the antimicrobial activity of the aglycone fraction obtained by acid hydrolysis of crude phenolic extract of cucumber cotyledons from seedlings treated for 48 h with 3.1 nmol of P1, P2, or P3 (bars 2, 3, and 4, respectively) or *Trichoderma* spores (bar 1) before a challenge with *P. syringae* pv. *lachrimans*. The bioassay was performed with *P. syringae* pv. *lachrymans* as the test microorganism. Untreated plants (bar 9) or plants treated with peptides (bars 6, 7, and 8) or bacteria only (bar 5) served as controls. The diameter of the lytic zone was measured. Each column represents the mean inhibition diameter of three independent experiments ± the standard deviation. *, significantly different from the other treatments (one-way factorial ANOVA; $P < 0.01$).

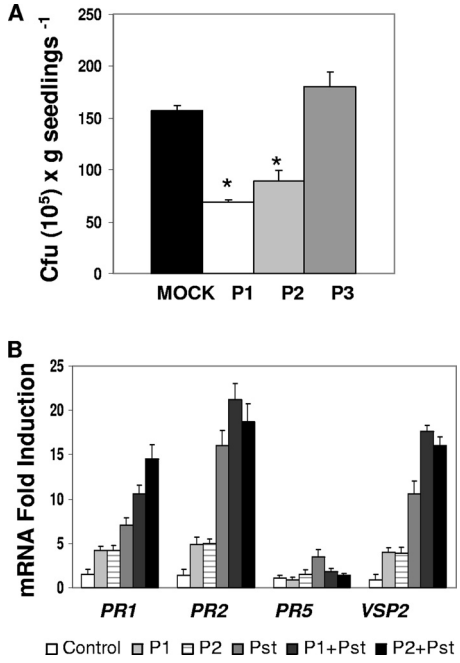

FIG. 4. Induction of defense responses in *Arabidopsis* seedlings by ultrashort lipopeptides. (A) Inhibition of *P. syringae* DC3000 multiplication. Fifteen 1-week-old seedlings were dispensed into each well of a 12-well tissue culture plate with 1 ml of B5 medium. Seedlings were treated with 1.6 nmol of the different peptides for 24 h before bacterial infection (10^4 CFU/ml). CFU counts of surviving bacteria were assayed 48 h after inoculation. *, significantly different from the other treatments (one-way factorial ANOVA; $P \ll 0.001$). (B) Induction of plant defense genes by synthetic peptides P1 and P2. Fifteen 1-week-old seedlings were dispensed into each well of a 12-well tissue culture plate with 1 ml of B5 medium. Seedlings were treated with 1.6 nmol of the different peptides for 24 h before bacterial infection (10^4 CFU/ml). Total RNA was extracted 24 h after a bacterial challenge from a pool of 15 seedlings/treatment and used for quantitative RT-PCR analysis of the expression of the pathogenesis-related protein-encoding genes *PR1*, *PR2*, and *PR5* and the jasmonic acid-responsive gene *vsp2*. *n*-fold induction of mRNA was compared to that in untreated seedlings (Control), to that in seedlings infected with the bacteria (*P. syringae* pv. *tomato* [Pst]) without previous peptide treatment, or to that in seedlings treated with peptides (P1 and P2) but not infected with bacteria. The results are averages of six independent replicates/treatment ± the standard errors tested by one-way factorial ANOVA, followed by TukeyHSD with $P < 0.05$ in comparison to the controls.

the application of 3.1 nmol of peptide at the roots and a challenge with the foliar bacterium *P. syringae* pv. *lachrimans*. The antimicrobial plate assay shows clear zones of bacterial inhibition in fractions extracted from plants pretreated before a bacterial challenge with peptides P1 and P2 but not before a bacterial challenge with peptide P3 (Fig. 3B). Application of 3.1 nmol of peptide alone did not induce significant antimicrobial activity in leaf extracts, nor did the application of bacteria alone. We did not find antibacterial activity in untreated plants. As a control for the system, cucumber seedling roots were treated with spores of *T. asperellum* T203, a beneficial fungus which is known to induce antimicrobial activity in aglycon fractions in cucumber plants (49) (Fig. 3B).

Induction of defense responses in *Arabidopsis* seedlings. *Arabidopsis* seedlings were infected with a suspension of the pathogenic bacterium *P. syringae* DC3000. Again, peptides P1 and P2 ($P < 0.001$), but not P3, afforded protection when 1.6 nmol of each peptide was applied 24 h prior to a bacterial challenge (Fig. 4A). Cocultivation of bacterial suspensions with peptides at the same concentrations did not inhibit bacterial growth (data not shown), demonstrating that the previously observed reduction in bacterial growth was due to defense responses activated in the plants.

Upregulation of several defense response-related genes by

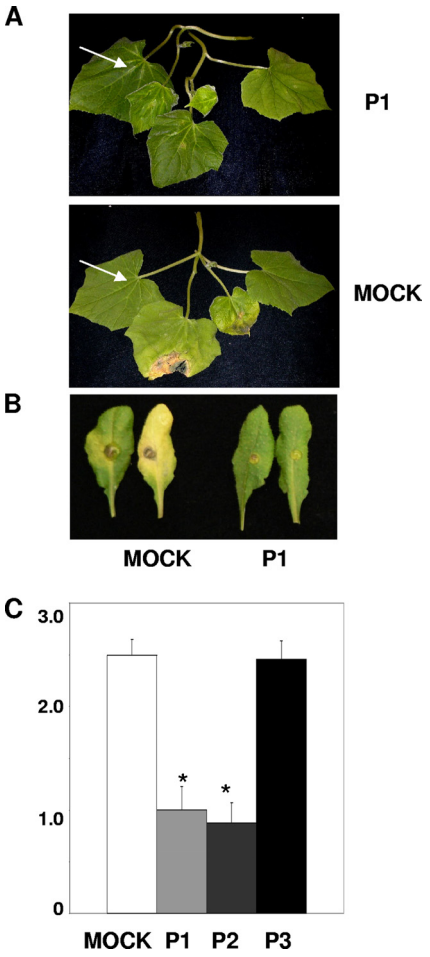

FIG. 5. Systemic protection against *B. cinerea* B05 infection by ultrashort peptides in cucumber and *Arabidopsis* seedlings. (A) The first leaf of cucumber plants (white arrows) was infiltrated with 100 μl of water (MOCK) or 100 μl of a 12.5 μM solution of P1 24 h before inoculation of the second and third leaves with *B. cinerea* mycelium. Symptoms were assessed 3 to 4 days after infection. (B) *Arabidopsis* rosette leaves 3 to 4 days after infection with *B. cinerea* spores. Four leaves from each plant rosette were treated with 100 μl of water (MOCK) or 100 μl of 12.5 μM peptides. Pathogen inoculation was done on nontreated leaves. Three to 4 days later, infected leaves were detached and analyzed. (C) *Arabidopsis* disease indexes, ranging from 0 (no symptoms) to 3 (severe lesions), were assessed according to the disease symptoms of 48 leaves in three independent experiments. Bars indicate standard deviations. *, significantly different from the other treatments. G-test-based analysis revealed the dependence of the observed disease index and the peptide tested ($P \ll 0.001$).

peptides P1 and P2 was monitored by quantitative PCR (Fig. 4B). We observed a significant increase in the expression of the gene *vsp2* ($P < 0.01$) following pretreatment with either peptide prior to a bacterial challenge compared to that in samples from plants treated with the pathogen only (Fig. 4B). P2 pretreatment also induced significant upregulation ($P < 0.01$) of the *PR1* gene.

Systemic protection against the pathogenic fungus *B. cinerea* BO5. Leaves of cucumber or *Arabidopsis* plants were infiltrated with peptides P1, P2, and P3 or water as a control. After 24 h, neighboring plant leaves that had not been directly treated were infected with a *B. cinerea* inoculum. Symptoms were assessed 3 to 4 days later. Figure 5A and B show results for systemic protection induced by peptide P1. G-test-based analysis revealed a dependence between the observed disease index and the peptide tested ($P \ll 0.001$). As shown in Fig. 5C, both peptides P1 and P2, but not P3, induced systemic protection in this assay.

DISCUSSION

We recently reported that short, positively charged peptides attached to fatty acids of various lengths are highly potent inhibitors of plant-pathogenic fungi and bacteria in vitro and also in planta, when applied directly at the infection area, without harming plant tissues (23). At the applied doses, the lipopeptides act via lysis of the pathogen membrane (22, 23). The most important finding in the present study is that some of these ultrashort lipopeptides are also inducers of systemic plant defense responses. Interestingly, the most active lipopeptides, P1 and P2, are not necessarily the short lipopeptides with the highest or broadest in vitro activity (23). It is also noteworthy that two peptides with longer peptidic chains, P6 and P7, although potent killers of bacteria (24, 34), were practically inactive in ISR induction assays (Fig. 1). One possible explanation for this is the different capacities of the plant to take up the different peptides. Different uptake capacities could also influence the true active concentration of the peptides, which might be much lower than the applied dose.

Lipopeptides have a membranolytic mode of action in bacteria and fungi; hence, the active peptides likely induce transient membrane perturbations also in plants. These, in turn, could activate signaling cascades leading to plant defense activation. Changes in membrane potential are the initial responses in many signaling pathways (12). Several signal peptide molecules that activate both defensive and developmental plant genes have been previously shown to induce alkalinization in cell suspension cultures when added at nanomolar concentrations (13, 36). The medium alkalinization assay performed on tobacco cells indeed suggests that peptides P1 and P2, and to a minor extent also P3, can induce pH changes (Fig. 2). Alkalinizing activity was induced in the tobacco cell suspension bioassay within minutes after addition of the lipopeptides and showed kinetics similar to those induced by other signal peptides (17).

Early responses to elicitors in cell suspension cultures, such as extracellular medium alkalinization, were suggested to be linked to the development of ISR in whole plants, although not always with a strong correlation (44). Here we saw a direct correlation between lipopetide activities in medium alkalinization and induction of plant defense responses with regard to both gene expression (Fig. 3A and 4B) and antimicrobial compound synthesis (Fig. 3B). However, the peptides are still capable of inducing an effective ISR at concentrations with less pronounced effects on pH. It should also be noted that these peptides have direct antifungal/antibacterial activities at micromolar concentrations (23), and therefore we should consider the possibility that their protective effects are also due to peptide migration through the plant from inoculated roots to infected leaves. Nonetheless, our experiments suggest that the peptides enhance the defense response signaling of the plant and do not act directly. A feature common to ISR responses

induced by beneficial microorganisms or their effectors is priming for enhanced defense. In primed plants, defense responses are not activated directly but are accelerated upon pathogen or insect attack, resulting in enhanced resistance to the attacker (46). The inhibitory activity of the aglycone fraction from extracts of plants treated only with the peptides (or pathogen) is significantly lower than that of the extracts from plants pretreated with the peptides and then challenged with the pathogen (Fig. 3B). Gene expression studies with cucumber plants also suggest that expression of specific genes is higher in pathogen-challenged plants pretreated with the lipopeptides (Fig. 3A).

ISR inducers vary greatly and include fungi, bacteria, viruses, nematodes, insects, components and products of pathogens and nonpathogens, organic and inorganic polymers, and simple organic and inorganic compounds. Since so many unrelated agents can elicit ISR, activation of a common mechanism in the plant is far more probable than the possibility of a common structure or composition (19). In this work, we provide new evidence that synthetic ultrashort lipopeptides, similar to *Bacillus subtilis* cyclic lipopeptides (31), are capable of inducing defense signaling pathways in plants and systemic protection to foliar bacterial and fungal diseases in both cucumber and *Arabidopsis* plants.

Direct application of AMP to plant surface organs was shown to be successful, albeit with a limited protective effect if the pathogen escapes the AMP application area (24). Overexpression of transgenes encoding natural or synthetic antimicrobial proteins and peptides has been demonstrated as a successful approach to protect plants against diseases caused by microorganisms (8, 15, 20, 21, 33, 50). However, low stability due to small size and susceptibility to protease degradation of the peptides is a problematic aspect of transgenic expression in plants.

Induction of systemic resistance in crops is an attractive protective strategy because it can activate defenses throughout the whole plant. Because so many different types of stimuli can be involved in the induction of the process and since its implementation may involve cross talk among several defense pathways, pathogen resistance to plant systemic mechanisms may be less likely to develop (1). The successful application of synthetic fully biodegradable and low-cost AMPs to plant protection may help in eradicating certain plant diseases and reducing the environmental impact of intensive agriculture.

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