Plants are constantly exposed and threatened by a variety of pathogenic microorganisms present in their environments. Diseases caused by pathogens, including bacteria, fungi, and viruses, significantly contribute to the overall loss in crop yield worldwide (31, 44, 51). In an effort to combat diseases, plants have devised various mechanisms and compounds to fend off microbial invaders. One of the most ancient and widespread defense strategies involves the production of cysteine-rich antimicrobial peptides (AMPs) composed of 45 to 54 amino acids (13, 14, 20, 37, 57). AMPs have been shown to be produced by a wide range of organisms and serve as their natural defense against infection (60, 61). Moreover, plant AMPs show structural and functional homology to their counterparts in animals, insects, and mammalian AMPs (8), whose involvement in host defense is well established (23, 46, 61). Many plant AMPs can rapidly inhibit the growth of a broad range of fungi and bacteria at micromolar concentrations, being nontoxic to both insects, and mammalian AMPs (8), whose involvement in host defense is well established (23, 46, 61). Many plant AMPs can rapidly inhibit the growth of a broad range of fungi and bacteria at micromolar concentrations, being nontoxic to both mammalian and plant cells (10, 39). Plant AMPs include α-defensins, lipid transfer proteins, thionins, hevein, and knottins (7, 10, 49). They are expressed either in a constitutive and organ-specific manner, in which they are most abundant in peripheral cell layers of the organ, or in a pathogen-modulated manner, possibly associated with altered tissue specificity (7, 45, 53). The locations of AMPs at the peripheries of different organs, their induction under pathogenic stress conditions, and their in vitro and in planta antimicrobial activities support the notion that plant AMPs are important components of the plant's defense system (8).

Despite the existence of defense mechanisms, plants are exposed to attack by fungi and bacteria. Fungi attack leaves, stems, fruits, and flowers before and after harvest. Some of the most common plant-pathogenic fungi belong to the genera Alternaria, Botrytis, Cochlisbolus, Fusarium, Geotrichum, Penicillium, and Sclerotina (6). These fungi cause considerable loss of crop yields worldwide. Moreover, many potentially human-pathogenic fungi and the yeast Saccharomyces cerevisiae have their natural habitats in the environment, including on plants and food items (55). In support of that, human-pathogenic fungi have been isolated from spoiling fruits. In addition, some fungi, such as Fusarium spp. and Alternaria spp., produce mycotoxins that can seriously harm consumers of fruit (50).

Plant fungicides based on synthetic chemicals are extensively used in agriculture. In fact, there are now more than 113 active ingredients registered as fungicides worldwide (26). However, they cause severe and long-term environmental pollution, are highly and acutely toxic, and are sometimes even carcinogenic toward humans and wild animals (12). Furthermore, pathogens become resistant to many of these chemicals (12, 43). Note also that some of the antimycotic compounds, such as members of the azole family, which are widely applied for plant protection, have derivatives that either are used medically or are under clinical evaluation (59). Therefore, it is anticipated that their extensive use in agriculture will increase the risk of selection of resistance phenotypes of human-pathogenic fungi from the surrounding environment or directly from the fungal flora of the human consumer during prolonged exposure to the compounds that are used both in agriculture and in medicine (5).
In addition to fungi, pathogenic bacteria are also responsible for great losses in crops (54). Yet, a major difficulty encountered is the lack of effective control against some severe diseases. Plant protection against these pathogens is mainly based on copper derivatives and antibiotics, which are environmentally contaminants. Furthermore, resistant strains of bacteria have already been reported (19). A very destructive pathogen is the gram-negative bacterium *Pseudomonas syringae*, which grows in the intercellular spaces of plant tissues (36).

Consequently, there is an obvious need to search for alternative compounds that are nontoxic to animals and nonpolluting environmentally to control fungal and bacterial plant diseases for agricultural applications, especially compounds with activity against phytopathogenic fungi and bacteria that acquire resistance to commercial compounds.

Membrane-active compounds are under investigation as a promising group. Their membrane lytic mechanism makes it difficult for the microorganisms to develop resistance (23, 47, 52, 61). These compounds include peptides and their synthetic derivatives mimicking gene-encoded AMPs from various species as well as lipopeptides, cyclopeptides, and cyclic lipopeptides (CLPs) which are produced via nonribosomal synthesis by various microorganisms (3, 31–35, 40–42). In most cases, the use of synthetic peptides is limited due to high-cost production (11, 19). Therefore, CLPs have received considerable attention for their broad antimicrobial properties against plant pathogens. They are produced by several plant-associated and soil-inhabiting bacteria, such as *Pseudomonas* and *Bacillus* spp. (3, 34, 35). Note that some CLPs and lipopeptides from microbial origins, such as syringomycin and syringopeptin, have significant phytotoxicities that limit their direct use as plant protection products (31). However, several others, such as surfactin and fengycin, have no phytotoxicity (38).

Recently, we have reported on a new family of ultrashort lipopeptides with a potent and broad spectrum of in vitro antimicrobial activity against human-pathogenic yeast, fungi, and bacteria. These lipopeptides are composed of only four L- and N-amino acids linked to fatty acids. The sequence of the peptidic moiety and the length of the fatty acyl group determine the specificities of the lipopeptides against bacteria, fungi, and mammalian cells (28). Here, we found that several ultrashort lipopeptides exert potent antifungal and antibacterial activities toward phytopathogenic fungi and bacteria in vitro and in planta. Their probably low phytotoxicities, drastic lytic modes of action, and natural amino acid compositions make them attractive candidates for future antibiotics in agriculture.

### MATERIALS AND METHODS

**Materials.** Rink amide 4-methyl benzhydrylamine resin and 9-fluorenylmethoxy carbonyl (Fmoc) amino acids were obtained from Calbiochem-Novabiochem AG (Switzerland). Lauric acid (dodecanoic acid) was purchased from Thermo Fisher Scientific AG (Switzerland). Lauric acid (dodecanoic acid) was purchased from Sigma Chemical Co. (Israel). Other reagents used for peptide synthesis included HCl (Sigma), and D-amino acids linked to fatty acids. The sequence of the peptide at room temperature. 

**Fungal and bacterial strains and culture conditions.** *Botrytis cinerea* strain BC03 (vine pathovar from I. Chet, Israel) were maintained on potato dextrose agar (PDA) at room temperature. *Cochliobolus heterostrophus* strain C4 (ATCC 48331) was maintained on complete medium at room temperature under continuous light (48). *Pseudomonas syringae* pv. tomato strain DC3000 (resistant to rifampin) was maintained on solidified Trypticase soy broth at 28°C. *Agrobacterium tumefaciens* (strain HGL0) was maintained on LB medium at 28°C.

**Peptide synthesis, acylation, and purification.** Peptides were synthesized by an Fmoc solid-phase method on Rink amide 4-methyl benzhydrylamine resin by using 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 (RP-HPLC; >98%) as described previously (2). The lipopeptide compositions were confirmed by electrospray mass spectrometry and amino acid analysis.

**In vitro antifungal activity.** The antifungal activities of the lipopeptides were examined in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 200 μl as follows. One hundred microliters of a spore suspension containing *B. cinerea*, *A. alternata*, or *C. heterostrophus* at a concentration of 2 × 10^7 CFU/ml in potato dextrose broth was added to 100 μl of potato dextrose broth containing the lipopeptides in serial twofold dilutions. The fungi were incubated at 25°C for 48 h using a Binder KB 115 incubator. Growth inhibition was determined by measuring the absorbance at 750 nm with an E1309 microplate autoreader (Biotek Instruments). The antifungal activity is expressed as the MIC, the concentration at which no growth was observed.

To further evaluate the antifungal effect of the lipopeptides, inhibition of hyphal growth was observed under an inverted light microscope (Eclipse TE300; Nikon, Tokyo, Japan) at 48 h postinoculation. Abnormalities in the morphologies of spores and hyphae were observed and photographed with a digital camera (C7424-95; Hamamatsu, Bridgewater, NJ) attached to the phototub of the microscope.

**In vitro antibacterial activity.** The antibacterial activities of the lipopeptides were examined in sterile 96-well plates (Nunc F96 microtiter plates) in a final volume of 100 μl as follows. Aliquots (50 μl) of a suspension containing bacteria at concentrations of 10^8 CFU/ml in culture medium were added to 50 μl of double-distilled water containing the lipopeptide in serial twofold dilutions in LB. Inhibition of growth was determined by measuring the absorbance at 600 nm with an E1309 microplate autoreader (Biotek Instruments) after an incubation of 48 h at 28°C. The antibacterial activity was expressed as the MIC, the concentration at which no growth was observed after the incubation.

**Antifungal assay on detached cucumber leaves infected with *B. cinerea*.** Cucumber seedlings (Kfr variety; Gedera Seeds Ltd., Israel) were grown in soil in 250-ml pots in a controlled environment. Leaves were detached from 2- to 3-week-old plants and were placed in closed plastic containers on wet filter paper to maintain high humidity. Detached cucumber leaves were inoculated with 5-mm-diameter mycelial agar discs of *B. cinerea* taken from 7- to 10-day-old cultures on PDA, with the disc (~2 × 10^6 spores) placed in the middle of each leaflet (16). The containers were placed in a climate chamber at 22°C. After 24 h, the discs were removed and 30 μg/ml of the lipopeptides was applied to the leaves. The lesion development was followed for 4 days after infection and 3 days after treatment. For antifungal treatment, the leaves were first sprayed with 30 μg/ml lipopeptides, and after 24 h, the leaves were inoculated with *B. cinerea* as described above. The lesion development was followed for 3 days after treatment.

**Antifungal assay on cucumber fruits infected with *B. cinerea*.** Cucumbers were purchased from local supermarkets in Rehovot, Israel. Wet filter paper was placed on the bottoms of closed plastic containers to maintain high humidity. Two or three fruits were placed on the bottom of each plastic container. A small part of the fruit was removed from the central upper part, and a 5-mm-diameter mycelial agar disc of *B. cinerea* (~2 × 10^6 spores) taken from 7- to 10-day-old cultures on PDA was placed in the middle of each fruit. The containers were placed in a climate chamber at 22°C. The mycelial agar discs were removed at 24 h postinoculation, and the fruits were sprayed immediately with 30 μg/ml of the lipopeptides. The treatment was repeated two more times every 24 h. The inoculum development was followed for 3 days after the last treatment.

**Antifungal assay on corn seedlings infected with *C. heterostrophus*.** Corn seedlings (sweet corn; Escalibur) were grown in soil in 100-ml pots in a controlled environment for 1 to 2 weeks. Inoculation of *C. heterostrophus* on corn plants was performed by spotting onto the surfaces of corn leaves 20-μl drops (0.1% Tween in double-distilled water) of spore suspensions (2.5 × 10^6 spores/mL) taken from 7- to 10-day-old culture on complete medium. Corn seedlings were covered with plastic bags overnight and maintained at room temperature. Then, at 24 h postinoculation, the corn leaves were sprayed with 30 μg/ml of C14-KL1K. The inoculum development was followed for 3 days after treatment.

**Antifungal assay on cucumber fruits infected with *C. heterostrophus*.** Cucumbers were purchased from local supermarkets in Rehovot, Israel. Wet filter paper was placed on the bottoms of closed plastic containers to maintain high humidity. Two or three fruits were placed on the bottom of each plastic container. A small part of the fruit was removed from the central upper part, and a 5-mm-diameter mycelial agar disc of *C. heterostrophus* (~2 × 10^6 spores) taken from 7- to 10-day-old cultures on PDA was placed in the middle of each fruit. The containers were placed in a climate chamber at 22°C. The mycelial agar discs were removed at 24 h postinoculation, and the fruits were sprayed immediately with 30 μg/ml of the lipopeptides. The treatment was repeated two more times every 24 h. The inoculum development was followed for 3 days after the last treatment.

**Antifungal assay on corn seedlings infected with *C. heterostrophus*.** Corn seedlings (sweet corn; Escalibur) were grown in soil in 100-ml pots in a controlled environment for 1 to 2 weeks. Inoculation of *C. heterostrophus* on corn plants was performed by spotting onto the surfaces of corn leaves 20-μl drops (0.1% Tween in double-distilled water) of spore suspensions (2.5 × 10^6 spores/mL) taken from 7- to 10-day-old culture on complete medium. Corn seedlings were covered with plastic bags overnight and maintained at room temperature. Then, at 24 h postinoculation, the corn leaves were sprayed with 30 μg/ml of C14-KL1K. The inoculum development was followed for 3 days after treatment.
TABLE 1. MICs of the lipopeptides against phytopathogenic fungi and bacteria

<table>
<thead>
<tr>
<th>Peptide designation</th>
<th>Plant-pathogenic fungi</th>
<th>Plant-pathogenic bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alternaria alternata</td>
<td>Botrytis cinerea</td>
</tr>
<tr>
<td>C16-KLLK</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td>C14-KLLK</td>
<td>12.5</td>
<td>3.1</td>
</tr>
<tr>
<td>C12-KLLK</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>C16-KKKK</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>C16-KGKK</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>C16-KA-KK</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>C14-KA-KK</td>
<td>100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>C16-GFGK</td>
<td>100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

* a Amino acids in italics are the D-enantiomers.

Antibacterial assay on Arabidopsis thaliana plant leaves infected with P. syringae. Five-week-old A. thaliana ecotype Colombia plants grown under a 10-h photoperiod at 21°C were sprayed with a bacterial suspension of P. syringae pv. tomato strain DC3000 diluted with 10 mM MgCl2 plus 0.02% Silwet L-77 to an optical density at 600 nm corresponding to 0.025 S × 10^7 CFU/ml. At 4 h preinoculation or 4 h postinoculation, 30 μg (100 μl) of peptide was infiltrated to rosette leaves by using a syringe without needle. To obtain 100% relative humidity, after inoculation the plants were covered with nylon. Two days after inoculation, the plants were assayed for bacterial colonization by grinding the rosette leaves in 10 mM MgCl2 and plating bacteria on Kings B agar plates after inoculation, the plants were covered with nylon. Two days after inoculation, the plants were assayed for bacterial colonization by grinding the rosette leaves in 10 mM MgCl2 and plating bacteria on Kings B agar plates (24) with rifampin (100 μg/ml).

Light microscopy of B. cinerea-infected cucumber leaves. Detached cucumber leaves were inoculated with B. cinerea as described above. After 24 h, the leaves were sprayed with 50 μl C14-KLLK. After an additional 24 h, discs (diameters of 10 mm) were cut out, fixed, and discolored in FAA solution (formalin-ethanol-acetic acid; 1:1:1). After being stained with methyl blue solution (lactophenol cotton blue; 0.05%), the leaf discs were observed under a light binocular. Images were obtained using a digital camera attached to a Leica MZ16FA stereomicroscope (DC300F; Leica Microsystems).

Evaluation of cytotoxicity by a peptide infiltration assay. Different concentrations of melittin or C14-KLLK (100 μl) were infiltrated into the mesophylls of fully expanded tobacco leaves with a syringe without a needle. The appearance of symptoms on the leaves was followed for 48 h after infiltration.

Examination of spores and hyphal damage by transmission electron microscopy. Samples containing B. cinerea spores (3.5 × 10^7 CFU/ml) or C. heterostrophus mycelium were incubated with or without the lipopeptides at their MICs for 20 min. The fungi were fixed by incubation with 1% glutaraldehyde in phosphate-buffered saline (4°C) for 1 h, then with 1% osmium tetroxide in phosphate-buffered saline containing 5% methyl blue solution at 24 h posttreatment (Fig. 3, panel 2). A drop containing the fungi was dehydrated by 20% carbon-coated grid and negatively stained with 1% uranil acetate. The grids were examined on a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan).

Enzymatic degradation. Trypsin (0.05%) was added to a solution of the lipopeptides in phosphate-buffered saline (4 μM), and the reaction was monitored by using RP-HPLC and electrospray mass spectroscopy. We used a C18 reverse phase Vydac analytical column (250 by 4 mm, 300-Å pore size, 7-μm particle size). The column was eluted for 40 min using a linear gradient of 40 to 80% acetonitrile in double-distilled water containing 0.05% trifluoroacetic acid (vol/vol) at a flow rate of 0.6 ml/min. Electrospray mass spectroscopy was performed to confirm the compositions and molecular weights of the products.

RESULTS

We have synthesized a series of very short lipopeptides composed of four L- and D-amino acids linked to aliphatic acids with different lengths. The sequence of the peptidic moiety was KXXX (X designates L, A, G, or K). A peptide comprising the four N-terminal amino acids of the AMP magainin served as a negative control. All of the peptides were amidated at their C termini, and one of their amino acids was replaced with a D-enantiomer. Table 1 lists the lipopeptides investigated. In order to test the stability of the lipopeptides toward enzymatic degradation, they were treated for 2 h with 0.05% trypsin, and the solution was analyzed by using RP-HPLC and mass spectroscopy. The data reveal that the lipopeptides were fully degraded by the enzyme (data not shown).

In vitro antifungal and antibacterial activity. The in vitro antifungal activities of the lipopeptides were assayed against different species of phytopathogenic fungi that are common in plant fungal infections and are known as major causes of disease in agriculture crops. These include Alternaria alternata, Botrytis cinerea, and Cochliobolus heterosporus. Their MICs are listed in Table 1. The data reveal that only the Leu-containing lipopeptides have potent antifungal activity, with C14-KLLK being the most active lipopeptide. The lipopeptides were also assayed against two different species of plant-pathogenic bacteria known as major sources of plant disease. These include Pseudomonas syringae and Agrobacterium tumefaciens; their MICs are listed in Table 1. The data reveal that most of the lipopeptides have antibacterial activity.

Sporophores and hyphal-growth anomalies due to the presence of the lipopeptides. Examination by use of light microscopy (Fig. 1) revealed that C14-KLLK, at its MIC, induced large-scale damage to spores and hyphae of A. alternata, B. cinerea, and C. heterostrophus. The effects of the lipopeptides on spores of B. cinerea and C. heterostrophus hyphae were also visualized by using transmission electron microscopy (Fig. 2). The fungi were incubated for 20 min with C14-KLLK at concentrations corresponding to the MICs. Significant differences in the morphologies of the treated fungi were observed. More specifically, the cell membrane and the cytoplasm of C. heterostrophus hyphae shrank after treatment with C14-KLLK, finally detaching from the rigid cell wall (Fig. 2B). Similarly, C14-KLLK caused a disruption of a large portion of the B. cinerea spor membrane and cell wall that led to leakage of intracellular material, which led to shrinkage of the cytoplasm and cell membrane from the rigid cell wall (Fig. 2D).

Inhibition of fungal infection in planta. We infected leaves of corn seedlings with the pathogen C. heterostrophus and detached cucumber leaves and fruits with gray mold (B. cinerea). The lipopeptides were then applied above the infected leaves and fruits. The results with B. cinerea were in agreement with the in vitro assay (Fig. 3, panel 1). The fungal infection expansions were followed for 4 days after infection and 3 days after treatment. Expanded black necrotic lesions were observed in the control (untreated) leaves. In contrast, the lesion expansions were totally inhibited in infected leaves that were treated with 30 μg/ml C14-KLLK. The same treatment with C16-KLLK was less effective. Similar results were obtained for cucumber leaves that were treated with 30 μg/ml C14-KLLK 24 h prior to infection with B. cinerea conidia (data not shown).

We further examined B. cinerea hyphal growth in infected leaves untreated or treated with C14-KLLK and stained with methyl blue solution at 24 h posttreatment (Fig. 3, panel 2). The data revealed dramatically reduced growth of hyphae in the treated leaves compared with that in the untreated leaves. Moreover, the hyphae in the treated leaves exhibited very thin and undeveloped structures compared with the hyphae in the untreated leaves. In addition, a few nongerminated spores could be detected in the treated leaves.

The antifungal activity of C14-KLLK (three consecutive
treatments) was also tested on cucumber fruits infected with *B. cinerea*. As shown in Fig. 3 (panel 3), the lesion’s expansion was fully inhibited. Further examination of slices of the cucumber revealed that the internal part of the untreated cucumber had severe fungal infection, whereas no infection was observed in the internal part of the treated cucumber. A similar in planta antifungal treatment was done on corn seedlings infected with *Cochliobolus heterostrophus* (Fig. 3, panel 4), and the lipopeptide prevented the expansion of the necrotic lesions.

**Inhibition of Pseudomonas syringae growth in vivo by C14-KLLK.** The in vivo antibacterial activity of C14-KLLK was investigated in *Arabidopsis* plants that were inoculated by spraying them with the common bacterial phytopathogen *P. syringae*. The *Arabidopsis* leaves were treated by infiltrating the

![Microscopic images of fungi 48 h after treatment or without treatment with the lipopeptides. Panel designations are as follows. For *Alternaria alternata*: A, blank; B, with 6 μM of C14-KLLK. For *Botrytis cinerea*: C, blank; D, with 6 μM of C14-KLLK. For *Cochliobolus heterostrophus*: E, blank; F, with 6 μM of C14-KLLK. Panels B-1, D-1, and F-1 are ×4 magnifications of panels B, D, and F, respectively.](http://aem.asm.org/)
ultrashort lipopeptide into the mesophyll at a concentration of 30 μg/ml 4 h before or 4 h after inoculating the leaves with the bacteria. Both of these treatments with C14-KLLK displaced similar antibacterial activities in vivo, which is manifested by the significant reduction (sixfold) in the number of bacterial cells in the treated leaves compared with the number in the untreated leaves (Fig. 4).

**Toxicity of C14-KLLK against plant tissues.** The toxicity of C14-KLLK against plant tissues was assessed by infiltrating 120 μg/ml, 60 μg/ml, and 30 μg/ml of the lipopeptide solution into the mesophylls of tobacco leaves (Fig. 5). Melittin, a nonspecific, 26-amino-acid, non-cell-selective peptide, was used as a positive control and caused cell necrosis. In large areas of leaves, mesophyll, infiltrated by melittin, became progressively clearer, and 12 h after infiltration, this area started to desiccate. The necrotic area then turned brown and was completely desiccated at 24 h postinfiltration. In contrast, no necrosis could be detected in the mesophylls of the leaves that were infiltrated with the lipopeptide C14-KLLK.

**DISCUSSION**

In the present study, we demonstrated that short, positively charged peptides attached to fatty acids of various lengths are potent inhibitors of plant-pathogenic fungi and bacteria, in vitro and in planta, without harming plant tissues. The most
active lipopeptides are those with the peptidic moiety KLKL. Interestingly, the lengths of the fatty acids did not have a major effect on the activities of the corresponding lipopeptides C16-KLKL and C14-KLKL against the different pathogens (Table 1). In comparison, previous studies have shown that the lipopeptide C16-KLKL, with a long palmitic acid, is less effective on human-pathogenic gram-negative bacteria than on gram-positive bacteria, whereas the lipopeptide C14-KLKL, with a shorter myristic acid, is equally active on both types of bacteria (29). Note that the bacteria selected are P. syringae, which causes disease symptoms ranging from leaf spots to stem cankers (36), and A. tumefaciens, which causes crown gall disease (18). Importantly, the MICs of these lipopeptides are similar to or higher than those of many long AMPs, particularly taking into account their relatively low molecular masses (~600 Da). Owing to the superior activities of the C14-KLKL lipopeptides on the various pathogens, we used these lipopeptides for further in vitro mode-of-action studies as well as for the in planta experiments.

Our data revealed that lipopeptides act via lysis of the pathogen membrane. This was demonstrated by both light microscopy (Fig. 1) and transmission electron microscopy (Fig. 2). At its MIC, C14-KLKL induces severe membrane disruption and lytic activity toward all the examined spores as well as growth inhibition of the fungal hyphae (Fig. 1). Furthermore, in both treated C. heterostrophus hyphae and B. cinerea spores, membranes and cytoplasms shrank and separated from the rigid cell wall, possibly due to loss of intracellular liquid (Fig. 2). Importantly, the drastic lytic effect is rapid (20-min incubation time), which should make it difficult for the pathogen to develop resistance. Nevertheless, the proposed effects on the membrane may also be partially a result of the cell wall disruption rather than a direct effect of the lipopeptides, due to the thick cell walls of these pathogens.

The lipopeptides are also active in vitro against Pythium aphanidermatum (data not shown). This microorganism belongs to the oomycete family, a unique group of diploid, fungus-like organisms related to chromophyte algae and other heteokont protists (4), which do not contain or produce sterols (1). This strong inhibitory activity (data not shown) further supports the notion that the lipopeptides directly act on and disrupt the membranes of the microorganisms without the involvement of sterols and ergosterol, which have been shown to affect the activity of the antifungal polyene family (9, 21).

Many studies were conducted with AMPs against plant pathogens in vitro and in planta, and their advantages and disadvantages are discussed in the introduction (3, 11, 15, 31–35, 40–42). Here, we demonstrated the successful application of the ultrashort lipopeptides as fungicides for treatment of gray mold on different plant tissues. Briefly, we infected cucumber leaves with mycelial agar discs of B. cinerea, and after 24 h of incubation, we treated the mycelium with C14-KLKL and C16-KLKL (Fig. 3, panel 1). C14-KLKL efficiently and
completely inhibited the expansion of the fungal mycelium in the cucumber leaves, whereas C16-KLLK did so to a lesser extent, in agreement with the in vitro results. Interestingly, treatment with C14-KLLK 24 h prior to infection with fungi also prevented the establishment and expansion of the fungal mycelium on the cucumber leaves. This demonstrates that the lipopeptide can conserve its antifungal activity for at least 24 h after being administered to the plant leaves. Further analysis of the fungal mycelium in leaf tissue stained with methyl blue solution revealed the inhibition of growth of the fungal hyphae as well (Fig. 3, panel 2). Furthermore, the diameter of the hyphal cylinder was also dramatically reduced compared to that in the untreated tissue. Although the in vitro and in planta studies strongly support the membranolytic mechanism of these lipopeptides, other mechanisms, such as induced systemic resistance, could also contribute to the observed effect (17, 25, 27, 30, 38, 58).

The ability of the lipopeptide to also inhibit the establishment of mycelium in corn leaves infected with C. heterostrophus spores emphasizes the universal potant activity of C14-KLLK toward different fungi that infected different plants (Fig. 3, panel 4).

Losses particularly due to fungal invasions are much more significant for highly perishable fresh fruits and vegetables. The characteristics of fruits and vegetables, such as high levels of sugars and other nutrients, high moisture content, and low pHs, make them highly susceptible to attacks by different pathogenic fungi, and they are difficult to treat (6, 22, 55). In this study, we also showed that cucumber fruits infected with B. cinerea fully recovered after treatment with C14-KLLK (Fig. 3, panel 3). We further examined the lipopeptides’ toxicity by infiltrating high concentrations of the lipopeptides into the mesophylls of fully expanded tobacco leaves (Fig. 5). The effect of the lipopeptides was compared to that of melittin, a non-cell-selective lytic peptide, which induced necrotic and toxic properties in the plant cells (56). Infiltration of melittin resulted in hemolysis of the plant cells and comprehensive and severe damage to the leaf mesophyll tissues. In contrast, infiltration of the lipopeptides did not induce any toxicity in the mesophyll tissue.

Importantly, C14-KLLK was also active in planta against plant-pathogenic bacteria. P. syringae is a common and aggressive pathogen that grows in the intercellular spaces of plant tissues. Treatment with C14-KLLK of living Arabidopsis leaves infected with P. syringae efficiently and rapidly reduced the number of bacterial cells (Fig. 4).

In summary, we present a strategy for eliminating plant-pathogenic microorganisms by using very short and economically available antimicrobial lipopeptides. These lipopeptides, which are composed of natural building blocks, namely, amino acids and fatty acids, have an efficient and broad spectrum of activity toward both bacteria and fungi and act rapidly on the membranes of the microorganisms via a lytic mechanism by which it should be difficult to confer resistance. Moreover, the lipopeptides are biodegradable to natural proteases such as trypsin. Therefore, we can assume that, similarly to native plant AMPs, they will finally be cleaved by plant proteases and therefore might not cause environmental pollution. Due to the increasing resistance of plant pathogens to the currently available antimicrobial agents and the emerging need to eliminate toxic chemicals from agricultural use, these new ultrashort lipopeptides could serve as potential candidates to be developed as relatively economical broad-spectrum antifungal and antibacterial agents in agriculture.

ACKNOWLEDGMENT

This study was supported by the Joseph Cohn Minerva Center for Biomembrane Research. We thank Vladimir Kiss for his help with the fluorescence microscopy, Yehuda Markovits for electron microscopy studies, and Batya Zarmi for technical assistance.

REFERENCES
