A Potent Antimicrobial Peptide Derived from the Protein LsGRP1 of *Lilium*

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ABSTRACT

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LsGRP1 is a defense-related gene differentially expressed in lily leaves in response to pathogen attack. The difficulty in the expression of *LsGRP1* in *Escherichia coli* suggested the presence of antimicrobial activity in LsGRP1. To evaluate the antimicrobial trait of LsGRP1, three LsGRP1-derived peptides were chemically synthesized; namely LsGRP1^N (N-terminal region without the signal peptide), LsGRP1^G (glycine-rich region), and LsGRP1^C (C-terminal cysteine-rich region). LsGRP1^C was proposed to be a potential antimicrobial agent according to

its broad-spectrum and effective antimicrobial activity. LsGRP1^C displayed inhibition effects on bacterial and fungal growth, possibly by altering the integrity of the cell membrane, as indicated by scanning electron microscopy and SYTOX Green staining assays. Additionally, LsGRP1^C induced programmed cell death-like phenomenon in the tested fungal species as indicated by 2',7'-dichlorodihydrofluorescein diacetate and 4',6'-diamidino-2-phenylindole assays. Further immunofluorescence staining showed that LsGRP1^C was located at the fungal cell surface. According to these observations, we concluded that LsGRP1^C originated from the plant defense-related protein LsGRP1 would play a role as an antimicrobial peptide and have a potential for practical use.

Antimicrobial peptides (AMPs) present in living organisms are natural antibiotics that act as a primary defense barrier to prevent the invasion of pathogenic microorganisms (10,19). Plant AMPs are 3- to 10-kDa cationic peptides rich in cysteine or glycine residues. These plant AMPs expressing either constitutively or in response to pathogen attack are mostly localized in the extracellular matrix. The structure of plant AMPs is generally stabilized by cysteine-linked intramolecular disulfide bridges which ensure the effective interaction with microbial plasma membrane and lead to disruption of membrane integrity of the microorganism. Other antimicrobial mechanisms of AMPs in addition to membrane permeabilization have been proposed, such as suppression of nucleic acid and protein syntheses, inhibition of enzymatic activity, and induction of programmed cell death (5,8,20). Because AMPs exhibit broad-spectrum antimicrobial activity and diverse mode of actions, they are candidates highly recommended for drug development and agricultural application (4,18,25).

Induced resistance in plants refers to a state with enhanced defense in response to biotic and abiotic stresses (3,9,24,31). The plant hormone salicylic acid is a disease resistance modulator involving in defense signaling and stimulating the expression of numerous defense genes in many plant systems (1,13). *LsGRP1* (*Lilium* 'Stargazer' glycine-rich protein 1) is a defense-related gene of lily that increases expression post treatment with either salicylic acid or probenazole and inoculation with fungal pathogen *Botrytis elliptica* (Berk.) Cooke (6,15–17). *LsGRP1* encodes a glycine-rich protein of 138 amino acids (aa) with a 23-aa N-terminal signal peptide, which enables translocation of mature protein to the plasma membrane or extracellular matrix. The

deduced LsGRP1 sequence shares similarity with various plant GRPs and has a highly conserved domain structure, including a signal peptide (SP), a central glycine-rich domain, and a C-terminal cysteine-rich domain (6,15–17). In our previous observations, using several fusion protein expression systems to express LsGRP1 in *Escherichia coli* was problematic. The poor yield of fusion protein together with cell aggregation and cell death as indicated by other reports (2,7) suggested that LsGRP1ΔSP is toxic to *E. coli*.

In this study, the antimicrobial potential of LsGRP1ΔSP was interpreted by antimicrobial assays using three LsGRP1-derived peptides; namely, LsGRP1^N, LsGRP1^G, and LsGRP1^C, corresponding to N-terminal, glycine-rich, and cysteine-rich regions of the deduced LsGRP1, respectively. The mode of action of the LsGRP1-derived peptides exhibiting the most effective antimicrobial activity and the broadest antimicrobial spectrum was further investigated on different aspects such as the alterations of cell morphology and membrane permeability and the induction of programmed cell death.

MATERIALS AND METHODS

Microorganisms. The bacterial species used in this study included *Agrobacterium tumefaciens* C58C¹ (30), *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA), *Pseudomonas syringae* pv. *syringae* 61 (11), *Bacillus subtilis* 28-4 and *Xanthomonas campestris* XCP3 (both from Molecular Plant Pathology Laboratory in Department of Plant Pathology and Microbiology, NTU, Taipei, Taiwan, ROC). These bacterial species were cultured on Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar) and 523 medium (1% sucrose, 0.8% casein hydrolysate, 0.4% yeast extract, 0.2% KH₂PO₄, 0.03% MgSO₄ · 7H₂O and 1.5% agar; pH 7.0). The incubation temperatures for *E. coli* DH5α and the other bacterial species were 37 and 28°C, respectively. For antibacterial assay, bacteria were incubated in 3 ml LB broth at 180 rpm for 12 to 16 h and then diluted to a desired concentration using sterile deionized water.

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* The e-Xtra logo stands for "electronic extra" and indicates that Figures 3, 4, and 5 appear in color online.

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The fungal species used in this study included *Alternaria brassicicola* Ac1, *Colletotrichum acutatum* HL1 (both from Molecular Plant Pathology Laboratory in Department of Plant Pathology and Microbiology, NTU), *Botrytis cinerea* B-134 and *B. elliptica* B061 (both from Dr. Ting-Fang Hsieh in Plant Pathology Division, Taiwan Agricultural Institute, Taichung, Taiwan, ROC). *B. elliptica* B061 was cultured on V8 agar (20% V8 juice [Campbell Soup Company, Camden, NJ], 0.3% CaCO₃, and 1.5% agar) at 20°C for 5 to 8 days whereas the other fungi were cultured on potato dextrose agar (Difco Laboratories, Detroit) at 25°C for 7 to 10 days. Spore suspensions of different fungal species were prepared in sterile deionized water and diluted to a desired concentration.

Computational analysis. The antimicrobial activities of LsGRP1^N, LsGRP1^G, and LsGRP1^C (Fig. 1), which originated from LsGRP1 sequences (National Center for Biotechnology Information accession number AAL61539.1), were analyzed using AMP prediction servers AMPA (27), APD2 (32,33), AntiBP2 (14), CAMP (26), and ClassAMP (12).

Peptide preparation. The chemically synthesized (Genscript USA Inc., Piscataway, NJ) peptides LsGRP1^N, LsGRP1^G, and LsGRP1^C were purified by high-performance liquid chromatography using a solvent system composed of a mixture of acetonitrile, trifluoroacetic acid, and water, all at a purity >90%. The identity of synthetic peptides was confirmed by electrospray ionization mass spectrometry. Synthetic peptides were dissolved in sterile deionized water at a concentration of 10 mg/ml and stored at -20°C before use.

Growth inhibition assays. The antibacterial and antifungal activities of these peptides were determined by measuring the inhibition rates of bacterial growth and fungal spore germination. Sterile deionized water instead of the peptide solution was used as a control treatment.

Bacterial suspensions with an optical density at 600 nm of 0.15 were treated with peptide solutions for 90 min at the culture temperature of treated bacterial species, then serially diluted and plated on LB medium. After incubation for 20 to 24 h at their culture temperature, the CFU in each peptide-treated sample were quantified. Inhibition rate (%) = [(CFU in water treatment - CFU in peptide treatment)/CFU in water treatment] × 100%. On the other hand, fungal spore suspensions of 1 × 10⁵ or 5 × 10⁴ spores/ml were treated with peptide solutions at the culture temperature of treated fungal species for 16 to 20 h and then the spore germinations were examined under the microscope. A germ tube greater than two times the spore length was considered germinated. Inhibition rate (%) = [(number of spores germinating

in water - number of spores germinating in peptide solution)/number of spores germinating in water] × 100%. All assays were performed in triplicate. Based on the data, the concentrations of peptide for 50% growth inhibition (IC₅₀) of each microbe were determined.

Scanning electron microscopy. The morphological change of bacterial cells was examined by scanning electron microscopy (SEM). Cover slides precoated with 0.1% poly-L-lysine were deposited with 20 µl of a bacterial suspension of 10⁸ cells/ml and kept moist at 28°C for 12 h. The bacterial colonies attached to the cover slides were treated with 20 µl of peptide solution for 2 h. Then, the cells were fixed using 1% glutaraldehyde and washed three times with 150 mM phosphate buffer (pH 7.2), each for 15 min. Next, the cells on the slides were coated with 1% OsO₄ for 1 h, immersed in 2% uranyl acetate for 30 min, and washed three times with 150 mM phosphate buffer (pH 7.2), each for 15 min. The samples were dehydrated serially using 30, 50, 70, 85, 90, 95, and 100% (twice) ethanol, each for 30 min, and soaked twice in acetone, each for 30 min, then dried in CO₂ by using a critical point dryer HCP-2 (Hitachi, Tokyo) and coated with gold particles in an ion sputter E101 (Hitachi). Afterward, the prepared samples were examined under a scanning electron microscope (Inspect S; FEI Company, Hillsboro, OR).

SYTOX Green staining. The alteration of cell membrane permeability of bacterial and fungal cells was examined by SYTOX Green staining. Bacterial suspensions of 1 × 10⁸ cells/ml were treated with peptide solution in the presence of 1 µM SYTOX Green (Invitrogen) and incubated at 28°C and 180 rpm for 2 h before observation under a Leica DMR fluorescence microscope equipped with a Chroma Endow green fluorescent protein filter set (a 450 to 490 nm band-pass excitation filter, a 495 nm dichroic mirror, and a 500 to 550 nm band-pass emission filter). Fungal spores and hyphae were treated with peptide solution at 50 µg/ml for 2 and 16 h, respectively. Then, the fungal cells were stained with SYTOX Green at 1 µg/ml in the dark for 10 min before observation under a Leica DMIL fluorescent microscope equipped with a Chroma 41012 filter set (a 460 to 500 nm band-pass excitation filter, a 505 nm dichroic mirror, and a 510 nm long-pass emission filter).

2',7'-Dichlorodihydrofluorescein diacetate staining. The accumulation of reactive oxygen species (ROS) in the fungal cells in response to peptide treatment was examined by 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) staining. Fungal spores and hyphae were treated with peptide solution at 50 µg/ml for 2 and 16 h, respectively. Then, the fungal cells were stained with 10 µM H₂DCFDA in the dark for 60 min before observation under

LsGRP1 (accession number: AAL61539.1)			
Signal peptide 1 st - 23 th a.a.	LsGRP1 ^N 24 th - 49 th a.a.	LsGRP1 ^G 50 th - 100 th a.a.	LsGRP1 ^C 101 th - 138 th a.a.
LsGRP1-derived peptide			
Prediction server	LsGRP1 ^N	LsGRP1 ^G	LsGRP1 ^C
AMPA	+	+	+
APD2	-	+/-	+
AntiBP2	-	-	+
CAMP	-	+	+/-
ClassAMP	+	+	+

Fig. 1. Schematic pattern and antimicrobial activity prediction of LsGRP1-derived peptides. LsGRP1^N, LsGRP1^G, and LsGRP1^C are indicated on different blocks of the deduced LsGRP1 sequence. Antimicrobial activities of LsGRP1-derived peptides predicted by server AMPA (27), APD2 (32), AntiBP2 (14), CAMP (26), and ClassAMP (12) are shown below. A putative antimicrobial activity was predicted (+) or not predicted (-); +/- indicates an ambiguous result predicted by the server.

a Leica DMIL florescent microscope equipped with a Chroma 41012 filter set.

4',6'-Diamidino-2-phenylindole staining. The programmed cell death phenomenon of the fungal cells in response to peptide treatment was examined by 4',6'-diamidino-2-phenylindole (DAPI) staining. Fungal spores and hyphae were treated with peptide solution at 50 µg/ml for 2 and 16 h, respectively. Then, the fungal cells were stained with DAPI at 1 µg/ml in the dark for 10 min before observation under a Leica DMIL florescent microscope equipped with a Leica A filter set (a 340 to 380 nm band-pass excitation filter, a 400 nm dichroic mirror, and a 425 nm long-pass emission filter).

Immunofluorescence microscopy. To examine the location of peptide molecules on the fungal cells treated, immunofluorescence microscopy was performed. At first, the fungal hyphae treated with peptide solution at 50 µg/ml for 16 h were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄; pH 7.4) for 1 h, treated with 25 mM dithiothreitol in PBS for 20 min, then digested with chitinase at 20 mg/ml in PBS containing β-mercaptoethanol at 5 µl/ml for 30 min. Afterward, the hyphae were immunostained using LsGRP1^C antibody and fluorescein isothiocyanate-labeled anti-rabbit immunoglobulin G antibody (KPL, Gaithersburg, MD). The hyphae were observed under a Leica DMIL florescent microscope equipped with a CHROMA 41012 filter set. LsGRP1^C antibody was prepared from the antiserum of the rabbit immunized with chemically synthesized partial C-terminal sequences of the deduced LsGRP1 (accession

number AAL61539.1) by purification with the LsGRP1^C affinity column.

RESULTS

Prediction of antimicrobial activity. LsGRP1^N, LsGRP1^G, and LsGRP1^C were assessed for antimicrobial activity using different AMP prediction servers. Antimicrobial activity of LsGRP1^C was suggested by all five servers used whereas antimicrobial potentials of LsGRP1^N and LsGRP1^G were only supported by two and four servers, respectively (Fig. 1).

Antibacterial activity of LsGRP1-derived peptides. In an antibacterial assay, the growths of five bacterial species were significantly inhibited in the presence of LsGRP1-derived peptides at a concentration of 2.5 mg/ml (Fig. 2A). Both LsGRP1^N and LsGRP1^C caused >99.5% growth inhibition on all bacterial species tested. Although LsGRP1^G caused >99.5% inhibition on the growth of *A. tumefaciens* C58C¹, it showed a lower inhibitory effect on *B. subtilis* 28-4 (87.6%), *E. coli* DH5α (81.8%), *P. syringae* pv. *syringae* 61 (84.8%), and *X. campestris* XCP3 (69.6%). Thus, LsGRP1^N and LsGRP1^C exhibited higher growth inhibition abilities on the bacteria compared with LsGRP1^G.

Antifungal activity of LsGRP1-derived peptides. The antifungal activities of the LsGRP1-derived peptides were investigated by measuring the inhibition rate of spore germination, and LsGRP1^C exhibited the highest inhibition activity and broadest spectrum compared with LsGRP1^N and LsGRP1^G (Fig. 2B). At a concentration as high as 2.5 mg/ml, LsGRP1^C inhibited spore

Fig. 2. Analysis of antimicrobial activity of LsGRP1-derived peptides. **A**, Bacterial suspension with an optical density at 600 nm of 0.15 was treated with peptide solution at 2.5 mg/ml for 90 min, and the CFU in each peptide sample were measured at 20 to 24 h post treatment by serial-dilution plating. Inhibition rate (%) = [(CFU in water treatment – CFU in peptide treatment)/CFU in water treatment] × 100%. **B**, Fungal spores with a concentration of 1×10^5 spores/ml were treated with peptide at 2.5 mg/ml for 16 to 20 h before examination of spore germination. Inhibition rate (%) = [(number of spores germinating in water – number of spores germinating in peptide solution)/number of spores germinating in water] × 100%.

germination by >90% in all assayed fungal species: *A. brassicicola* Ac1, *B. cinerea* B-134, *B. elliptica* B061, and *C. acutatum* HL1. On the other hand, LsGRP1^N inhibited spore germinations of *A. brassicicola* Ac1 and *C. acutatum* HL1 by 98.0 and 40.3%, respectively. The spore germination of *B. cinerea* B-134 was not affected by LsGRP1^N. Worthy of notice, LsGRP1^N slightly enhanced spore germination of *B. elliptica* B061 (–12.4% inhibi-

TABLE 1. The 50% inhibitory concentrations (IC₅₀) of LsGRP1^C for bacterial and fungal species

Microorganism	IC ₅₀ (μg/ml)
Bacterial species^a	
<i>Agrobacterium tumefaciens</i> C58C ¹	71.79
<i>Bacillus subtilis</i> 28-4	23.32
<i>Escherichia coli</i> DH5α	9.67
<i>Pseudomonas syringae</i> pv. <i>syringae</i> 61	20.63
<i>Xanthomonas campestris</i> XCP3	32.19
Fungal species^b	
<i>Alternaria brassicicola</i> Ac1	54.80
<i>Botrytis elliptica</i> B061	86.13
<i>Botrytis cinerea</i> B-134	58.26
<i>Colletotrichum acutatum</i> HL1	17.39

^a Inhibition of bacterial growth was measured (initial optical density at 600 nm = 0.15).

^b Inhibition of fungal spore germination (5 × 10⁴ spores/ml) was measured.

tion rate). In contrast to LsGRP1^N and LsGRP1^C, LsGRP1^G did not inhibit spore germination, with the exception of *B. elliptica* B061 (62.7% inhibition rate). Hence, LsGRP1^C is a more potent antifungal peptide compared with LsGRP1^N and LsGRP1^G.

LsGRP1^C exhibited effective inhibition on bacterial and fungal species. To further demonstrate the antimicrobial activity of LsGRP1^C, the IC₅₀ values of LsGRP1^C on different bacterial and fungal species were determined. The IC₅₀ values of LsGRP1^C on the assayed microbes were all <90 μg/ml (Table 1). With the exception of *A. tumefaciens* C58C¹ (IC₅₀ = 71.79 μg/ml), the IC₅₀ values of LsGRP1^C on all assayed bacterial species were <35 μg/ml, with *E. coli* DH5α being most sensitive to LsGRP1^C (IC₅₀ = 9.67 μg/ml). On the other hand, the IC₅₀ values of the assayed fungal species were <60 μg/ml, except that the causal pathogen of lily leaf blight, *B. elliptica*, exhibited higher tolerance to LsGRP1^C (IC₅₀ = 86.13 μg/ml).

LsGRP1^C altered bacterial morphology and membrane permeability. The effects of LsGRP1^C on the morphology of two plant-pathogenic bacteria, *P. syringae* pv. *syringae* 61 and *X. campestris* XCP3, were examined by SEM. After co-incubation with LsGRP1^C at 25 μg/ml, the cells of *P. syringae* pv. *syringae* 61 and *X. campestris* XCP3 exhibited a shortened, wrinkled, and melted morphology (Fig. 3A). When the concentration of LsGRP1^C treatment increased to 250 μg/ml, the surface of treated

Fig. 3. Changes in morphology and membrane permeability of bacteria treated with LsGRP1^C. **A**, Observation of *Pseudomonas syringae* pv. *syringae* 61 and *Xanthomonas campestris* XCP3 by scanning electron microscopy post LsGRP1^C treatment. Spherical structures on the rod-shaped bacterial cells were indicated by arrows. Bar = 4 μm. **B**, SYTOX-Green-stained *P. syringae* pv. *syringae* 61 and *X. campestris* XCP3 post LsGRP1^C treatment. Bar = 10 μm.

cells became coarser, with some knurls and bubble-like appearance. At a concentration of LsGRP1^C as high as 2,500 µg/ml, a significant number of spherical particles of different sizes were observed and some of them had a budding appearance. Besides, the spherical particles were associated with collapsed cells in a rod shape. In addition, to verify the effect of LsGRP1^C on bacterial membranes, a nucleic acid dye, SYTOX Green, impermeable to normal cells but penetrating to damaged cell membranes and causing fluorescent nuclei, was used. The SYTOX Green-stained bacterial cells were observed in *P. syringae* pv. *syringae* 61 and *X. campestris* XCP3 after LsGRP1^C treatment (Fig. 3B) whereas the control without LsGRP1^C treatment did not show the fluorescent cells in the assayed bacterial species, indicating that LsGRP1^C destroyed the integrity of bacterial membranes.

LsGRP1^C destroyed membrane integrity and induced programmed cell death-like phenomenon in fungi. The effects of LsGRP1^C on spores and hyphae of *A. brassicicola* Ac1, *B. cinerea* B-134, *B. elliptica* B061, and *C. acutatum* HL1 were demonstrated (Fig. 4). As observed under a light microscope, the spores and hyphae of LsGRP1^C-treated fungal species, especially *B. elliptica*, the causal pathogen of lily leaf blight, exhibited bleb and shrinkage on the cell surface as well as necrotic and granular in the cytoplasm. In contrast, the surfaces of the fungal spores and hyphae in the control were smooth and intact, and the cytoplasm of different assayed fungi all had a homogeneous feature without granulation and necrosis.

Damage to the plasma membrane of spores and hyphae caused by LsGRP1^C appeared in all fungal species examined. In addition, DAPI staining showed that LsGRP1^C treatment led to nuclear chromatin condensation in these fungal species examined (Fig. 4). The accumulations of ROS in the hyphae of the fungi pretreated with LsGRP1^C were visualized by H₂DCFDA staining (Fig. 4B). These effects of LsGRP1^C treatment as detected by SYTOX Green, DAPI, and H₂DCFDA staining were absent in the control. Interestingly, the ROS accumulation triggered by LsGRP1^C was absent in the ungerminated spores of the fungal species examined, except that of *B. elliptica* (Fig. 4A).

The location of LsGRP1^C on the treated fungal cells. LsGRP1^C-treated hyphae fluoresced in the fungal species tested whereas no signal was observed in the water control (Fig. 5). Most fluorescent signals were located at the outer layer of the hyphae and only a few were found inside the hyphal cells.

DISCUSSION

Cysteine and glycine are the most abundant amino acids in plant AMPs (10,32). The LsGRP1, a defense-related protein of *Lilium*, also contains cysteine- and glycine-rich regions. The cysteine-rich C-terminal region of LsGRP1, among the three regions evaluated, was selected by the prediction servers as the best candidate to possess antimicrobial activities.

Although the three LsGRP1-derived peptides effectively inhibited all of the bacterial species investigated (70 to 100%

Fig. 4. Effect of LsGRP1^C on plant fungal pathogens. **A**, Fungal spores and **B**, hyphae were treated with LsGRP1^C at 50 µg/ml for 2 and 16 h, respectively. Membrane permeability, chromatin condensation, and reactive oxygen species accumulation of treated spores and hyphae were assayed by staining with SYTOX Green, 4',6'-diamidino-2-phenylindole (DAPI), and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA), respectively. Sterile deionized water was used instead of LsGRP1^C as a control. Bar = 20 (A) or 50 (B) µm.

inhibition), only LsGRP1^C inhibited all the fungal strains investigated to a high degree as predicted by AMP prediction servers. The antimicrobial strength and spectrum of LsGRP1^C were further demonstrated by the low IC₅₀ values on the bacterial and fungal species examined. The IC₅₀ values of these LsGRP1^C-treated microbes were all <90 µg/ml, well within the effective range of known AMPs (19). The combined traits of effective inhibitory activity and broad antimicrobial spectrum of LsGRP1^C suggested that this peptide could act on certain common targets or has multiple effects on microbial physiology. The LsGRP1^C-treated cells of *P. syringae* pv. *syringae* 61 and *X. campestris* XCP3 exhibited a shortened and shrinkage feature at lower concentration and turned into a spherical shape at higher concentrations, indicating that the cell morphological changes of the two bacterial species examined were dose dependent. The morphological change of bacterial cells also implied that cell membrane damage might be involved and supported by SYTOX Green staining results. SYTOX Green is impermeable to normal cells but penetrates damaged cell membrane and stains the nucleic acid. The fact that SYTOX-Green-stained nucleic acids were observed in *P. syringae* pv. *syringae* 61 and *X. campestris* XCP3 cells treated with LsGRP1^C but not with the control indicated that LsGRP1^C changed the permeability of bacterial membranes by compromising the membrane integrity. Consistent with the dose-dependent morphological change, the proportion of SYTOX-Green-stained bacteria was positively correlated to the concentration of LsGRP1^C used in the treatment. The changes in

morphology and membrane permeability of bacteria triggered by LsGRP1^C are similar to the alterations that occurred in irreversible terminal differentiation of nitrogen-fixing bacteria into bacteroids, which is governed by AMP-like nodule-specific cysteine-rich peptides secreted by the inverted repeat-lacking clade of legumes during symbiosis (29). In addition, because the synthesis of peptidoglycan sacculus, the major contributor to maintain bacterial morphology, is associated with the bacterial membrane (28), it might be involved in the mechanism of bacterial morphology change induced by LsGRP1^C.

Damage to the plasma membrane of spores and hyphae by the treatment with LsGRP1^C demonstrated that LsGRP1^C also affected the membrane integrity of all fungal species tested. In the SYTOX Green staining assay, LsGRP1^C was shown to disrupt the membrane integrity of both bacterial and fungal cells, representing a common property of cationic cysteine-rich AMPs (5,20). Moreover, the predominant association of LsGRP1^C with the cell surface of fungal hyphae indicated that the location of the acting target of LsGRP1^C was primarily on the cell wall or plasma membrane. In addition to membrane permeability change, LsGRP1^C also caused nuclear chromatin condensation and ROS accumulation in the hyphae of all fungal species tested, indicating occurrence of the programmed cell death phenomenon of fungi (21). Because H₂DCFDA used for ROS detection is a membrane-permeant ionic molecule naturally trapped inside the cell, the efficiency of ROS staining would not be influenced by LsGRP1^C-triggered fungal membrane permeability. However, the ROS

Fig. 5. Immunofluorescence staining of LsGRP1^C on the hyphae of plant fungal pathogens. Fungal hyphae were treated with LsGRP1^C at 50 µg/ml for 16 h before immunofluorescence staining. Sterile deionized water was used instead of LsGRP1^C as a control. Bar = 25 µm.

responses were different in the fungal spores, suggesting that a different role was played by LsGRP1^C in the interaction between fungal spores and plants. Because only the spores of *B. elliptica*, a lily pathogen, exhibited an earlier ROS accumulation in response to the presence of LsGRP1^C compared with other tested fungal species, the higher sensitivity to LsGRP1^C of *B. elliptica* was probably due to host-specific recognition. Therefore, LsGRP1^C might not only directly alter membrane integrity in both bacterial and fungal microorganisms but also induce programmed cell death-like phenomenon, at least in some fungi. This feature was also observed in other antifungal peptides and proteins originating from different kinds of microorganisms, plants, and animals (8). Thus, the antimicrobial activity of LsGRP1^C might be a result of multiple effects on microorganisms.

LsGRP1 is a defense-related gene with an increased expression in lily leaves exhibiting salicylic acid-induced systemic resistance against *B. elliptica* (6,16), and it could enhance disease resistance *in vivo* when expressed in *Arabidopsis thaliana* and *Nicotiana benthamiana* (unpublished data). A number of plant AMPs and antimicrobial compounds have been suggested as inducers of programmed cell death in fungi (8,20,22,23). In this study, the *in vitro* inhibitory mechanism of LsGRP1^C on *B. elliptica*, a lily fungal pathogen, suggested that LsGRP1 might confer plant disease resistance *via* inducing fungal membrane permeabilization and programmed cell death. In contrast, the slight enhancement of spore germination in *B. elliptica* caused by LsGRP1^N treatment might be due to *B. elliptica* recognizing the corresponding region of LsGRP1 as a host signal resulting from the long-term coevolution of *B. elliptica* and lily. However, to understand the underlying mechanisms of the effects of different regions of LsGRP1 on *B. elliptica* will require further investigation.

LsGRP1^N, LsGRP1^G, and LsGRP1^C all exhibited high growth inhibition activities on both gram-positive and gram-negative bacteria, suggesting that all three regions might contribute to the unsuccessful expression of LsGRP1 protein in the *E. coli* system in our previous observation. In this study, the low IC₅₀ value of LsGRP1^C on *E. coli* DH5 α (9.67 μ g/ml) also indicated that *E. coli* would be particularly sensitive to the expressed LsGRP1-bearing C-terminal cysteine region. Because the antimicrobial potency of LsGRP1^C on different bacterial and fungal species was demonstrated herein, defense-related proteins that failed to express in a microbial system as LsGRP1 could be strong candidates as novel AMPs.

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