Evidence of Induced Systemic Resistance Against *Botrytis elliptica* in Lily

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Accepted for publication 18 February 2008.

ABSTRACT

Liu, Y.-H., Huang, C.-J., and Chen, C.-Y. 2008. Evidence of induced systemic resistance against *Botrytis elliptica* in lily. Phytopathology 98:830-836.

Lily leaf blight, caused by *Botrytis elliptica*, is an important fungal disease in Taiwan. In order to identify an effective, nonfungicide method to decrease disease incidence in *Lilium formosanum*, the efficacy of rhizobacteria eliciting induced systemic resistance (ISR) was examined in this study. Over 300 rhizobacteria were isolated from the rhizosphere of *L. formosanum* healthy plants and 63 were identified by the analysis of fatty acid profiles. Disease suppressive ability of 13 strains was demonstrated by soil drench application of bacterial suspensions to the rhizosphere of *L. formosanum* seedlings. Biocontrol experiments were carried out with *Bacillus cereus* and *Pseudomonas putida* strains on *L. formosanum* and *Lilium* Oriental hybrid cvs. Acapulco and Star Gazer in greenhouse and field studies. Plants treated with *B. cereus* strain C1L showed that protection against *B. elliptica* on *L. formosanum* could last for at least 10 days and was consistent with high populations of *B. cereus* on lily roots. Analysis of the expression of *LfGRP1* and *LsGRP1*, encoding glycine-rich protein associated with *L. formosanum* and cv. Star Gazer, respectively, revealed different responses induced by *B. cereus* or by the pathogen *B. elliptica*, suggesting that plant defense responses elicited by each follows a different signaling pathway. According to the results of biocontrol assays and *LfGRP1/LsGRP1* gene expression analyses with culture filtrates of *B. cereus* strain C1L, we propose that eliciting factors of ISR are generated by *B. cereus* and some of them exhibit thermostable and heat-tolerant traits. This is the first report about ISR-eliciting rhizobacteria and factors effective for foliar disease suppression in lily.

Botrytis leaf blight, caused by the fungal pathogen *Botrytis elliptica* (Berk.) Cooke, is one of the most important diseases of lilies (*Lilium* spp.) in Taiwan (2,13,19). Fungicides have been frequently used to control the disease in cut flower production, but effectiveness is limited due to the rapid development of fungicide resistance in *B. elliptica* (4,22). Other methods have shown potential to control Botrytis leaf blight of lily. Both *Bacillus amyloliquefaciens* B190 (5) and *Bacillus cereus* 28-9 (14), applied as foliar applicants, have shown effective in biologically controlling the disease. Similarly, systemic resistance activated by application with salicylic acid (SA) and probenazole has been demonstrated to be effective against lily leaf blight in *Lilium* Oriental hybrid cv. Star Gazer (16–18).

Biotic or abiotic factors can alter physiological or biochemical responses of plants to invoke multiple complex defense mechanisms. The biotic induction of plant responses by interactions with pathogenic or nonpathogenic microorganisms can result in systemic resistance responses referred to as systemic acquired resistance (SAR) (8,38) and induced systemic resistance (ISR) (1,15,29,40), respectively. Induction of SAR or ISR is elicited through different signaling pathways (27,45). However, enhanced resistance effective against a broad spectrum of plant pathogens, including fungi, bacteria, viruses, nematodes, and even insect herbivores, is a general phenomenon of systemic resistance triggered by different kinds of factors (38,40,46). Both kinds of induced resistances enhance plant innate defenses against subsequent biotic challenges (40). In the early state after pathogen attack, accumulation of endogenously synthesized SA triggers SAR activity (20,21), followed by the activation of genes encoding pathogenesis-related (PR) proteins (39,43). As demonstrated in *Arabidopsis thaliana*, prevention of SA accumulation by transgenic expression of *nahG* encoding for salicylate hydroxylase, results in no increased expression of *PR* gene and SAR activation, showing that SA is an important factor in the SAR signaling pathway (9). Since methyl salicylate (MeSA) esterase activity of the SA-binding protein 2 (SABP2) of tobacco can convert MeSA into SA, Park et al. inferred that MeSA acts as a crucial signal of SAR in tobacco (26). In contrast to SAR, ISR triggered in *A. thaliana* by *Pseudomonas fluorescens* WCS417r does not cause SA accumulation and PR gene expression, but instead requires the plant hormones jasmonic acid and ethylene to induce resistance in plants (27).

In previous studies, molecular analysis of gene expression in response to SA treatment in Oriental lily cv. Star Gazer identified a cDNA sequence encoding a 138-amino acid protein by suppression subtractive hybridization (17). This putative protein designated LsGRP1 shared homology with several glycine-rich proteins present in the plant extracellular matrix (17). Northern blot analysis detected an increase of *LsGRP1*-related mRNA in 'Star Gazer' leaves after SA treatment, and also in *B. elliptica*-infected and upper uninoculated leaves. When *B. elliptica* was inoculated onto SA-treated leaves, accumulation of *LsGRP1*-related mRNA appeared earlier compared with that without SA treatment, suggesting a possible role for LsGRP1 in the SAR in lily (17).

Lilium formosanum Wall. (Formosa lily) is a native lily species of Taiwan that is widely distributed mostly as a wild plant, but is also used for landscaping purposes (33,44). In 2003, an outbreak of Botrytis leaf blight severely devastated *L. formosanum* in Pulowan, Taroko National Park in Hua-lien, Taiwan. Based on previous observations demonstrating the potential of systemic resistance to control lily leaf blight in *Lilium* Oriental hybrid cv.

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doi:10.1094/PHYTO-98-7-0830
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Star Gazer (16–18), we examined whether ISR triggered by nonpathogenic rhizobacteria would be a good strategy to protect *L. formosanum* from infection by *B. elliptica*. To approach this goal, a screening experiment was performed to identify promising rhizobacteria from the rhizosphere of healthy *L. formosanum* and test their abilities to suppress Botrytis leaf blight on different kinds of lilies. In addition, we were interested in determining if glycine-rich proteins were associated with ISR in *Lilium* species. These results provide further insight into the mechanisms underlying SAR and ISR, especially in the monocot lily. In addition, characterization of the eliciting factor(s) of ISR was approached in the ISR-eliciting rhizobacterium *B. cereus* strain ClL.

MATERIALS AND METHODS

Bacterial and fungal strains and inocula preparation. The roots with least adhered soils were excised from *L. formosanum* plants appearing healthy in the landscape area of Pulowan, Taroko National Park in Hua-lien, Taiwan, and were subsequently stirred in sterile water for 30 min. Serial dilutions of bacterial suspensions were plated on Difco nutrient agar (Becton Dickinson) and incubated at 28°C. Isolates were purified to single colonies on Luria-Bertani (LB) agar medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar). Bacterial inocula for applications on plants were prepared by culturing strains in LB broth at 28°C for 16 h. Bacterial cells were harvested by centrifugation (4,000 × *g*, 10 min) and suspended in 10 mM $MgSO_4$ to a final concentration of 1×10^8 colony forming units (CFU)/ml. A solution of 10 mM $MgSO_4$ with no bacteria was used as a negative control.

B. elliptica strain B061-1 (17) was used for all experiments. Inoculum was prepared by growing the strain on V8 agar (20% V8 vegetable juice [Campbell soup group], 0.3% $CaCO_3$, and 1.5% agar) under near-UV light for 5 days (7,17,18). Conidia were collected by gently vortexing in Tween 20 solution (0.05% Tween 20 in sterile deionized water) and adjusted to a concentration of 5×10^4 conidia/ml as inoculum.

Identification of bacterial strains. Bacterial isolates were purified to single colonies several times on LB agar and identified by analysis of fatty acid profiles with the Microbial Identification System (MIDI, Inc., Newark, DE) following the protocol of Microbial Identification System, in that MIDI Sherlock software (version 5.0; MIDI, Inc.) coupled with ChemStation software (version 4.02; Hewlett-Packard, Avondale, PA). *B. cereus* 28-9 (14), a known strain from our lab, was used for comparison with new isolates.

Plant cultivation. For greenhouse studies, *L. formosanum* seedlings were grown in 7.5 cm pots (one seedling per pot) and the bulbs of Oriental lily cv. Star Gazer were grown in 14 cm pots (one bulb per pot) using potting mix consisting of peat moss and perlite at a ratio of 3:1. Plants were grown at 20 to 25°C with a cycle of 12 h/12 h light/dark for 30 to 45 days. For field assays, *L. formosanum* and Oriental lily cvs. Star Gazer and Acapulco plants were grown in the soil with individual plants 3.5 to 4 cm apart. Bacterial treatment began about 30 days after planting. The soil of sand-loam type was planted. Irrigation was applied by drenching twice a week.

Screening of bacterial strains based on ISR-eliciting potential and effect on plant growth. For screening the bacterial strains capable of eliciting ISR, the rhizosphere of *L. formosanum* seedling was drenched with 30 ml of bacterial suspension at 10-day intervals three times. *B. elliptica* was inoculated 4 days after the last bacterial application by atomizing conidial suspensions onto the abaxial surface of lily leaves until dripping off the leaves.

The extent of lesion development was scored 4 days after inoculation based on a disease rating scale from 0 to 4, in which 0 = no lesions; 1 = less than 12% of leaf area with lesions; 2 = 13 to 25% of leaf area with lesions; 3 = 26 to 50% of leaf area with lesions; and 4 = more than 50% of leaf area with lesions. Each treatment consisted of five plants and three leaves in each plant were inoculated. The effect of bacterial treatments on the growth of lily seedlings was assessed based on the alteration of leaf number.

Biological control assays. Biocontrol assays were performed in greenhouse conditions at National Taiwan University and under field conditions at Hua-lian, Taipei, and I-lan in North Taiwan. Bacterial strains tested included those selected from the rhizosphere of *L. formosanum* plants and *B. cereus* 28-9, a previously identified strain that demonstrated potential as a foliar applied biocontrol agent against *B. elliptica* (14). *L. formosanum* seedlings were used in the greenhouse assay. All other studies used adult plants. The rhizosphere of lily seedling was drenched with 30 ml of bacterial suspension, but 50 ml was used for adult lily plants. The time schedules of bacterial application were set as follows: (i) three times at 10-day intervals for greenhouse assay; (ii) eight times at 14-day intervals for *L. formosanum* plants in field studies; and (iii) three times at 10- and 14-day intervals for 'Star Gazer' and 'Acapulco', respectively, in field studies. *B. elliptica* was inoculated 4 days after the last bacterial application. The extent of lesion development on *L. formosanum* was scored based on a disease rating scale as described above, and the lesion number on Oriental lily was assessed. In the greenhouse assay on *L. formosanum*, each treatment consisted of five plants with three leaves per plant inoculated with the pathogen. Each treatment on Oriental lily cv. Star Gazer consisted of 10 plants, with three leaves of each plant inoculated with the pathogen. In field assays, each treatment consisted of 25 plants with five leaves per plant being inoculated. All experiments were repeated twice.

Bioassay of ISR-eliciting potential of *B. cereus*. To show the periods of ISR expression triggered by *B. cereus*, the rhizosphere of *L. formosanum* seedling was drenched with 30 ml of bacterial suspension on different days before fungal inoculation. The extent of lesion development was scored 4 days after fungal inoculation based on a disease rating scale as described above. Each treatment consisted of five plants with three leaves from each plant inoculated with the pathogen. The experiment was repeated twice.

In vivo rhizosphere colonization assay. Rhizosphere colonization ability of *B. cereus* strain C1L was studied using a rifampicin-resistant marker strain on the roots of *L. formosanum* seedlings. The rifampicin-resistant strain of *B. cereus* was isolated on LB medium containing rifampicin (100 mg/liter) according to the method previously described (28). Bacterial suspensions of the rifampicin-resistant strain were applied to the rhizosphere of lily seedlings as a soil drench (30 ml each). Rhizosphere samples were taken each day after suspensions were applied for the first 5 days, followed by samplings on 7 and 10 days after application. The top 1 cm of root, beginning from the stem, was dissected from three roots from each lily seedling and suspended in 1 ml of sterile water with vortexing for 10 min. Three seedlings were assessed at each time period after bacterial application. The suspension of root segments of a seedling was subjected to appropriate dilutions and plated on LB medium containing rifampicin (100 mg/liter). After growing overnight at 28°C, the number of bacteria on the root (per centimeter) at each time period after bacterial application was measured.

Assay of ISR-eliciting factors possibly produced by *B. cereus*. For investigation on the ISR-eliciting factors produced by *B. cereus* strain C1L, the rhizosphere of lily cv. Star Gazer was drenched with *B. cereus* culture filtrate, followed by fungal inoculation at 1, 4, and 7 days after pretreatment. Treatments consisted of (i) *B. cereus* strain C1L cell-free culture filtrates (through 0.45 μm filter) from 50 ml of 16 h culture in LB broth; (ii) *B. cereus* strain C1L culture filtrates autoclaved for 10 min; and (iii) uninoculated LB medium as a control. Each treatment consisted of five plants, with five inoculated leaves per plant. Three days

after fungal inoculation, lesion numbers were recorded. The experiment was repeated twice. In addition, *B. cereus* 28-9 was used as another test strain.

Cloning of *LsGRP1* homolog from *L. formosanum*. In order to analyze the gene expression in response to bacterial application in the rhizosphere of lily plants, a cDNA homolog of the known SA-, probenazole-, and pathogen-inducible *LsGRP1* of Oriental lily cv. Star Gazer (17,18) was cloned from *L. formosanum* and used as one of the DNA probes in northern blot hybridization analysis. Total RNA of the leaves of *L. formosanum* 48 h after inoculation with *B. elliptica* was extracted by a Plant Total RNA Extraction Miniprep System (Viogene, Sunnyvale, CA) and used to synthesize the cDNAs with PowerScript Reverse Transcript Kit (BD Bioscience Clontech, Heidelberg, Germany). Primers (5′-CCAAGGATCCGGTCGGGAGCTGGCTGAGGAAC-3′, 5′-CCAAGAATTCTTATGGGTGTCCATAAGCAGGCTC-3′) were designed according to the cDNA sequence of *LsGRP1* of Oriental lily cv. Star Gazer (accession no. AY072283) and used in the subsequent polymerase chain reaction (PCR) amplification of the *LsGRP1* homolog. The DNA fragments from reverse transcription-PCR were cloned into pGEMT easy vector (Promega, Madison, WI) and the resulting plasmids were transformed into *Escherichia coli* DH5α (Bethesda Research Laboratories). DNA sequencing was carried out with BigDye terminator cycle sequencing ready reaction in ABI310 DNA sequencer (Perkin-Elmer). Sequence data were analyzed for homology to *LsGRP1* using the basic local alignment search tool in the Blast program (provided online by the National Center for Biotechnology Information).

Northern blot hybridization analysis. The rhizosphere of lily plants was drenched with an equivalent amount of either *B. cereus* bacterial suspension (1.0×10^8 CFU/ml) or the culture filtrate at different periods before harvest. Suspensions of 30 and 50 ml were used for *L. formosanum* seedling and lily cv. Star Gazer plant, respectively. The leaves were detached and stored at –80°C until extraction. Total RNA of lily leaves was extracted and separated on 1% agarose gel prepared in 2.2 M formaldehyde-containing 1× MOPS buffer (40 mM MOPS, pH 7.0, 10 M sodium acetate, and 1 mM EDTA), using 1× MOPS running buffer. The membrane was UV cross-linked and hybridized with the respective digoxigenin (DIG)-labeled DNA probe that was prepared using PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Hybrids were detected with the DIG Luminescent Detection Kit for nucleic acids (Roche Diagnostics). In addition, lily leaves were inoculated with *B. elliptica* 1 day after application of bacterial suspension or culture filtrate, or without pretreatment, and examined for alteration in the amount of corresponding mRNA transcript.

Statistical analysis. The data obtained were subjected to the standard analysis of variance (ANOVA) procedure. Least significant difference values at $P = 0.05$ were used to separate treatment means when ANOVA indicated a significant F value.

RESULTS

Screening rhizobacteria isolated from the rhizosphere of *L. formosanum* for ISR-eliciting potential and positive effect on seedling growth. Over 300 bacteria were isolated from the rhizosphere of *L. formosanum* plants collected from Pulowan, Taroko National Park in Hua-lien, Taiwan. Sixty-three bacteria were identified by gas chromatography analysis of fatty acid methyl esters (Table 1). *Bacillus* and *Pseudomonas* species comprised 28 and 38% of all identified strains, respectively. The greenhouse screening test on *L. formosanum* seedlings showed that at least 13 bacterial strains were capable of decreasing lesion development on the leaves caused by *B. elliptica* when bacterial suspensions were applied to the rhizosphere. Among them, many showed a positive effect on plant growth but some had an adverse effect on plant growth (Table 1).

Biocontrol assays. In this study, *B. cereus* strains C1L and A3S21 and *P. putida* strains CF-3 and CF2-1 were compared with the previously characterized biocontrol strain *B. cereus* 28-9 (14) for their ability to suppress disease on lily. As shown in Table 2,

TABLE 2. Disease suppression by rhizobacterial strains against Botrytis leaf blight of *Lilium formosanum*[y]

Bacterial strain	Disease index[z]	
	Greenhouse assay	Field assay
Bacillus cereus C1L	0.8 bc	0.6 d
Bacillus cereus A3S21	1.3 b	1.3 b
Bacillus cereus 28-9	1.3 b	0.8 cd
Pseudomonas putida CF-3	1.3 b	0.9 bcd
Pseudomonas putida CF2-1	1.6 b	1.0 bcd
Disease CK	3.7 a	2.0 a
Health CK	0.0 c	0.0 e

[y] Suspensions of rhizobacteria (1×10^8 CFU/ml) were applied to the rhizosphere of lily plants three times at 10-day intervals in greenhouse assays, and eight times at 14-day intervals in field assay. Conidial suspensions of *Botrytis elliptica* were inoculated onto the abaxial surface of lily leaves 4 days after the final bacterial treatment.

[z] Values represent the average of disease index in a plant scored 4 days after fungal inoculation as described in the text. Greenhouse assay treatments consisted of five plants with three leaves inoculated per plant; field assay treatments consisted of 25 plants with five leaves inoculated per plant. Data in the same column followed by different letters are significantly different ($P = 0.05$).

TABLE 1. Bacterial strains isolated from *Lilium formosanum* rhizosphere and the screening tests

Species	Number of isolates	Biocontrol seedling test[y]	Strains showing positive effect[z]	Strains showing negative effect
Acinetobacter calcoaceticus	3	A3S10; A3S9	A3S10	A3S9
Arthrobacter viscosus	1			
Bacillus cereus	8	**C1L; A3S21**	C1L; A3S21	
Bacillus megaterium	8	**A3S34; A3S37**	A3S34; A3S37	
Bacillus pumilus	2	**A3S11**	A3S11	
Brevibacillus choshimensis	2	**A3S12; CF4-1-K**	A3S12	CF4-1-K
Burkholderia cepacia	7	**A3S36**; CF4-4-2; CFQP	A3S36; CF4-4-2	CFQP
Delftia acidov	1			
Pantoea agglomerans	2	**A3S15**		A3S15
Paucimonas lemoigenei	1			
Pseudomonas agarici	1			
Pseudomonas fluorescens	2	**A2S1**		A2S1
Pseudomonas putida	11	**CF-3; CF2-1; CF3-1**	CF-3; CF2-1	CF3-1
Pseudomonas syringae	10			
Rhizobium radiobacter	2			
Salmonella choleraesuis	2			

[y] Strains with good biocontrol potential are indicated in bold.

[z] Plant growth effect was assessed on the leaf number of *L. formosanum* seedlings.

these strains could protect *L. formosanum* from Botrytis leaf blight in both greenhouse and field conditions. The ability of the five strains to suppress disease was further tested on Oriental lily cultivars. Results indicated that these strains were able to decrease lesion number caused by *B. elliptica* on Oriental lily cvs. Star Gazer and Acapulco in the greenhouse and field studies, with *B. cereus* C1L exhibiting the best biocontrol activity (Tables 2 and 3).

ISR-eliciting potential of *B. cereus*. *B. cereus* strain C1L was assayed for ISR-eliciting activity and root colonization trait. Figure 1 indicates that a 40% reduction in disease severity could be achieved when *B. cereus* strain C1L bacterial suspension was applied to the rhizosphere of *L. formosanum* seedling 1 day before fungal inoculation. Such protection was further enhanced to 75% reduction of disease severity when the seedling was treated with *B. cereus* strain C1L bacterial suspension 4 or 5 days before fungal inoculation. About 70% of disease suppression could be maintained up to 10 days after bacterial application. To demonstrate the rhizosphere competence of *B. cereus* strain C1L, we used a rifampicin-resistant strain generated from *B. cereus* strain C1L to analyze bacterial colonization in the rhizosphere of *L. formosanum* seedlings. Results showed that *B. cereus* strain C1L populations were present at the top 1 cm of root beginning from the stem and that bacterial populations could be maintained at a level between 5×10^6 and 10^5 CFU/cm up to 5 days after bacterial application. *B. cereus* strain C1L was still present in the basal portion of the lily root at populations above 10^4 CFU/cm up to 10 days after application (Fig. 2).

Assay of putative ISR factors produced by *B. cereus*. Table 4 shows the effect of culture filtrates of *B. cereus* strains C1L and 28-9 on the infection of 'Star Gazer' leaves by *B. elliptica*. The culture filtrates of both strains applied to the rhizosphere of lily plants could decrease lesion number caused by *B. elliptica*; effective protection was achieved 1 day after treatment. Equivalent levels of disease suppression were observed when the culture filtrates were applied 4 and 7 days before fungal inoculation. When the culture filtrates of both *B. cereus* strains were autoclaved, their abilities to protect lily cv. Star Gazer from infection by *Botrytis elliptica* were maintained, especially when application was done 4 and 7 days before fungal inoculation. On the other hand, when treatment was carried out just a day before fungal inoculation, the autoclaved culture filtrates of *B. cereus* strain C1L and *B. cereus* 28-9 displayed much lower levels of protection

TABLE 3. Disease suppression by rhizobacterial strains against Botrytis leaf blight of Oriental lily cvs. Star Gazer and Acapulco[y]

	Number of lesions[z]		
	Greenhouse assay	Field assay	
Bacterial strain	'Star Gazer'	'Star Gazer'	'Acapulco'
Bacillus cereus C1L	7.8 b	0.8 d	2.6 c
Bacillus cereus A3S21	6.8 bc	6.5 ab	4.2 bc
Bacillus cereus 28-9	12.8 b	5.4 abc	4.5 bc
Pseudomonas putida CF-3	11.6 b	4.9 abc	12.1 b
Pseudomonas putida CF2-1	10.4 b	4.3 bc	4.4 bc
Disease CK	20.8 a	8.1 a	25.2 a
Health CK	0.0 c	0.0 d	0.0 c

[y] Suspensions of rhizobacteria were applied to the rhizosphere of lily plants three times at 10-day intervals for 'Star Gazer' and 14-day intervals for 'Acapulco'. Conidial suspensions of *Botrytis elliptica* were inoculated onto the abaxial surface of lily leaves 4 days after the last bacterial application. Lesion numbers were recorded 4 days after fungal inoculation.

[z] Values represent the average number of lesions on a leaf. Greenhouse assay treatments consisted of 10 plants with three leaves inoculated per plant; field assay treatments consisted of 25 plants with five leaves inoculated per plant. Data in the same column followed by different letters are significantly different ($P = 0.05$).

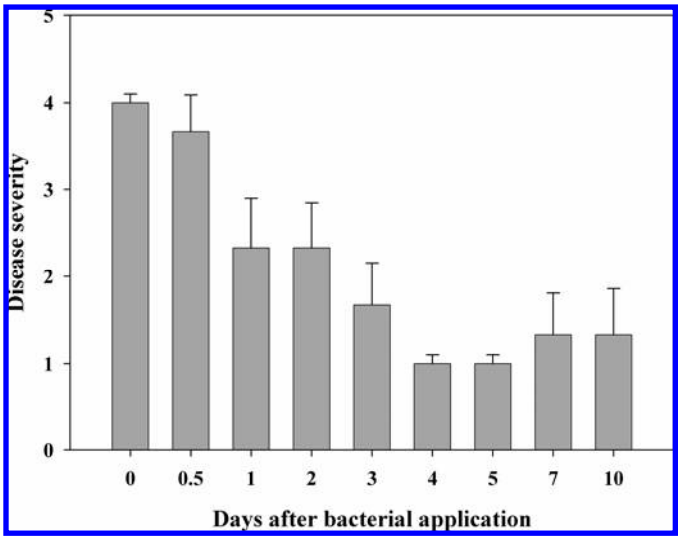

Fig. 1. Disease suppressing potential of *Bacillus cereus* strain C1L on *Lilium formosanum* over time. Fungal inoculation was performed different days after bacterial application to the rhizosphere of *L. formosanum*. Extent of lesion development was scored 4 days after fungal inoculation based on a disease rating scale. The value represents the average of disease index in a plant. Each treatment had five plants and three leaves in a plant were inoculated. Vertical bars indicate standard deviations of the mean.

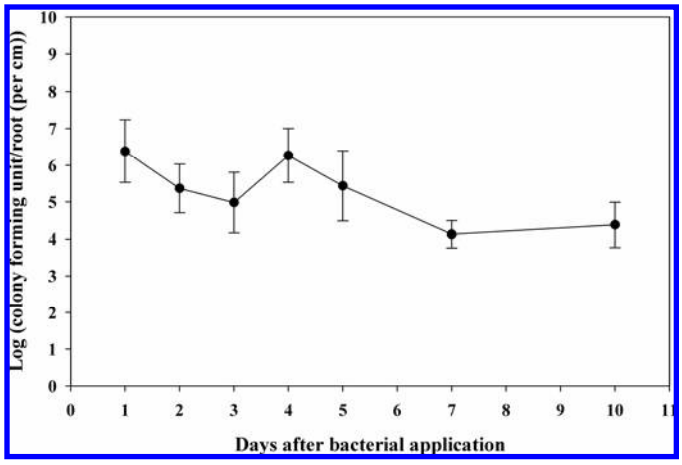

Fig. 2. Populations of *Bacillus cereus* strain C1L on the basal regions of *Lilium formosanum* roots over time. Value represents the mean of bacterial number from three root segments of a seedling. Different seedlings of three were used in each time point. Vertical bars represent standard deviations of mean values of bacterial number on the root (log(colony forming unit/root [per cm]).

TABLE 4. Disease suppression caused by the treatment of culture filtrates of *Bacillus cereus* strains against Botrytis leaf blight of Oriental lily cv. Star Gazer

Period of treatment before fungal inoculation[y]	Number of lesion[z]			
	B. cereus 28-9		*B. cereus* C1L	
	Un-autoclaved	Autoclaved	Un-autoclaved	Autoclaved
1 day	5.5 b	13.5 a	7.3 b	13.9 a
4 days	7.5 b	6.5 b	5.4 b	5.0 b
7 days	4.5 b	6.0 b	3.6 b	4.8 b
Disease CK	18.0 a	18.0 a	18.0 a	18.0 a
Health CK	0.0 c	0.0 c	0.0 c	0.0 c

[y] Culture filtrates of *B. cereus* strains in Luria-Bertani broth were applied to the rhizosphere of lily plants. Different periods after bacterial application, conidial suspension of *Botrytis elliptica* was atomized onto the abaxial surface of lily leaves.

[z] Values represent the average number of lesions on a leaf. Each treatment consisted of five plants with five leaves inoculated per plant. Lesion number was recorded 3 days after fungal inoculation. Data in the same column followed by different letters are significantly different ($P = 0.05$).

than the nonautoclaved ones, showing no significant difference in the lesion number compared with the LB-treated or untreated control leaves. Application of LB medium alone is not capable of decreasing symptom development as the culture filtrates of *B. cereus* strains.

Characterization and comparison of glycine-rich protein genes *LfGRP1* and *LsGRP1*. Plant glycine-rich proteins represent a diverse set of proteins that may interact with RNA in the nuclei, or to be associated with cell walls or membranes (30). A cDNA *LsGRP1* sharing homology with several glycine-rich proteins present in the plant extracellular matrix was cloned from Oriental lily cv. Star Gazer and used to show that the expression of *LsGRP1* is SA and *B. elliptica*-inducible (17). Using the primers designed to sequence encoding *LsGRP1*, an *LsGRP1* homolog was successfully amplified from *L. formosanum* and named *LfGRP1* (*Lilium formosanum* glycine-rich protein 1, accession no. EF681959), which encodes a protein of 135 amino acids. Nucleotide sequences of *LfGRP1* and *LsGRP1* shared 87% identity and the deduced amino acid sequences shared 86% identity. The deduced protein includes an N-terminal region rich in hydrophobic and nonpolar amino acids with a predicted cleavage site of signal peptidase between alanine (Ala-23) and glycine (Gly-24). The mature protein has a predicated molecular weight of 13.72 kDa and is rich in tyrosine (Y) (9.63%). A cysteine-rich (six in total) carboxyl terminus and a core region composed of glycine stretches, interspersed with tyrosine, proline (P), histidine (H), or asparagine (N) residues, were found. In total, the deduced amino acid sequence contained 28.89% of glycine residues, with two repeats of YQGGGGG, three repeats of YHNGGG, and one set of YPGGGG sequence, belonging to a type of glycine-rich repeat GGX1X2X3GG (30).

Necrotic lesions caused by *B. elliptica* on 'Star Gazer' leaves were decreased in plants treated with *B. cereus* strain C1L compared with untreated plants inoculated with the fungus (Fig. 3A). Increased expression of *LfGRP1* and *LsGRP1* in the leaves of *L. formosanum* seedlings and Oriental lily cv. Star Gazer plants, respectively, was observed 1 day after fungal inoculation and maintained for at least 7 days thereafter (Fig. 3B). In contrast, expression of *LfGRP1* and *LsGRP1* in *L. formosanum* and Oriental lily cv. Star Gazer, respectively, decreased 1 day after application of *B. cereus* strain C1L. Decreased expression of *LfGRP1* and *LsGRP1* transcripts was more pronounced in pathogen-inoculated plants beginning 1 day after *B. elliptica* inoculation, regardless of whether bacterial suspensions or culture filtrates were applied (Fig. 3C and D).

DISCUSSION

L. formosanum, a native species of lily, is used as a landscape plant throughout Taiwan. Outbreaks of leaf and flower blight have devastated *L. formosanum* planting in Hualien, a county of eastern Taiwan. Based on numerous reports of systemic resistance elicited by rhizobacteria in plants (1,15,29,40), we considered the feasibility of developing the method for reducing the prevalence of Botrytis blight in *L. formosanum*, especially in landscaped areas. Since good colonization of rhizobacteria in the plant rhizosphere would contribute to their ability to trigger systemic resistance (36), an attempt to isolate beneficial rhizobacteria from healthy stands of *L. formosanum* was carried out. Among 63 bacterial strains identified, the majority were found to belong to *Bacillus* or *Pseudomonas* species, implicating that the rhizosphere of *L. formosanum* is a good habitat for the species belonging to these groups. From the bacterial strains examined for potentials of inducing systemic resistance and effect on the growth of *L. formosanum* seedlings, two *B. cereus* and two *P. putida* strains were selected for further analysis of their biocontrol activity in this study. All four strains showed effective protection on *L. formosanum* against *B. elliptica* infection. It was noteworthy that *B. cereus* strain C1L consistently exhibited protection in the biocontrol experiments, as well as in greenhouse and field trials on different kinds of lilies.

Systemic resistance triggered by *Bacillus* species have been observed in many plants (15); however, *B. cereus* was first reported as a good biocontrol agent in lily by ISR induced through a mechanism that has yet to be determined. Other *Bacillus* species, such as *B. amyloliquefaciens*, have been screened from lily plants and identified to be suitable for protection by foliar application on Oriental lily against *B. elliptica* (5,6). Through seed microbialization, *B. cereus* was found to reduce disease severity of different kinds of fungal diseases (34,35). The ability of *B. cereus* strain C1L to induce systemic resistance in lily was substantially demonstrated herein using a soil drench application method.

B. cereus strain C1L was able to effectively colonize the *L. formosanum* roots, maintaining populations over 10^4 CFU/cm 10 days after application. Good colonization is a trait expected to facilitate ISR elicitation by rhizobacteria. For example, many rhizobacteria capable of eliciting ISR against *Pseudomonas syringae* pv. *tomato* could effectively colonize tomato roots (36). Other strains of *B. cereus* have been shown to maintain populations on host roots over extended periods. For example, *B. cereus* UW85 was shown to persist in the rhizosphere of soybean until harvest (10). Thus, it is possible that *B. cereus* strain C1L is capable of maintaining populations on lily roots for a period longer than 10 days, but such an assumption requires further examination.

According to the results shown in Figure 1, induction of systemic resistance in *L. formosanum* required a period of 1 day after bacterial application, after which the induced resistance effect could be maintained for at least 10 days, with an optimum observed 4 to 5 days after bacterial application. Effective protection by *B. cereus* strain C1L was manifested in Oriental lily cvs. Star Gazer and Acapulco, in addition to *L. formosanum*, suggesting the strain may be capable of inducing systemic resistance over a broad range of plant hosts, similar to *B. cereus* strain UW85 (11,12,25,37). The application of *B. cereus* strain C1L to other plant species of distant relationship is worthy of investigation.

Studies on ISR-eliciting factors have been approached in *Pseudomonas* spp. (1). Based on our observation of ISR phenomenon in lily against *B. elliptica* infection, exploration of the nature of elicitors produced by *B. cereus* strain C1L was initiated. Our results implicated that *B. cereus* C1L excreted factors that conferred activation of disease suppression in lily. After heat treatment by autoclaving, the timing of disease suppression remained similar to those caused by the original culture filtrates, except for the response in 1-day treatments. These results suggest both *B. cereus* strains C1L and 28-9 produce heat labile factors responsible for early elicitation of ISR observed within 1 day, and heat-stable factor contributing to ISR observed 4 days after treatment. However, it remains unclear whether the eliciting factors produced by *B. cereus* strains C1L and 28-9 are similar. Romeiro et al. (31) reported that *B. cereus* could release macromolecules to trigger systemic disease resistance in tomato. In view of the involvement of lipopeptides and volatile organic compounds of *Bacillus* species (24,32) in biological control, bacterial determinants responsible for the induction of systemic resistance become an intriguing subject of investigation in biocontrol strains of *B. cereus*.

LsGRP1 has been demonstrated as an SA-dependent, pathogen-inducible gene in Oriental lily cv. Star Gazer (17). In *L. formosanum*, expression of *LfGRP1* also exhibits pathogen inducibility, implying that similar defense responses might be triggered in different kinds of lilies. Negative regulation of gene expression in response to bacterial treatment has been demonstrated in nonpathogenic *Pseudomonas*-treated *Arabidopsis* (3,41,42) and *B. subtilis*-treated tomato and cucumber (23). Likewise, we found that both *LfGRP1* and *LsGRP1* transcripts decreased after application of strain C1L. Since expression of *LfGRP1* and *LsGRP1*

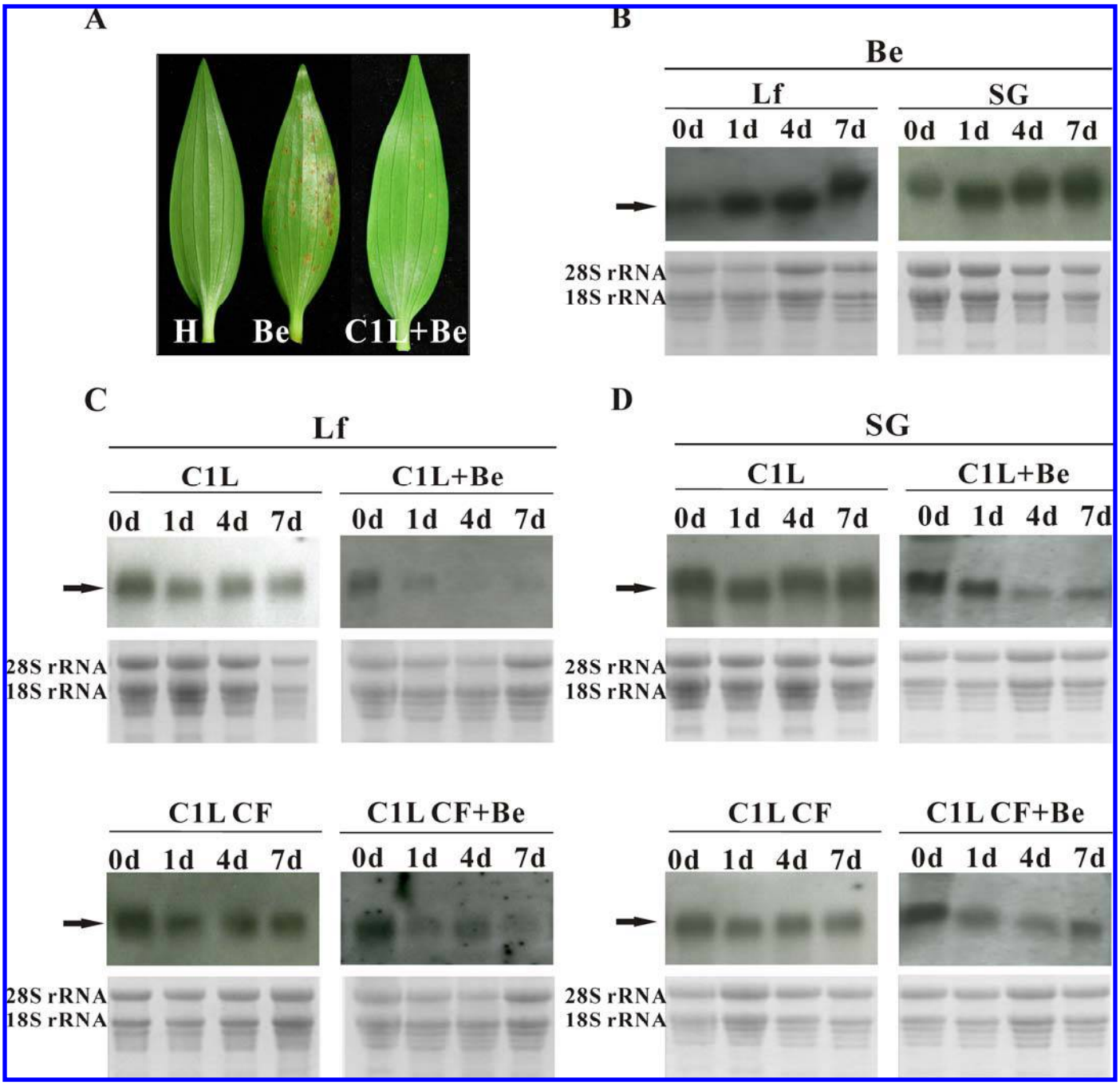

Fig. 3. Northern blot analyses of *LfGRP1* and *LsGRP1* expression in lily leaves in response to treatment with *Bacillus cereus* strain C1L. **A,** *Botrytis elliptica* was inoculated on 'Star Gazer' leaves 1 day after application of *B. cereus* strain C1L to the rhizosphere (C1L+Be) or without C1L treatment (Be). H, healthy leaves without fungal inoculation. Photograph was taken 3 days after fungal inoculation. **B,** Gene expressions of *LfGRP1* in *Lilium formosanum* (Lf) and *LsGRP1* in Oriental lily cv. Star Gazer (SG) were analyzed different days after fungal inoculations. **C and D,** Gene expressions of *LfGRP1* (Lf) and *LsGRP1* (SG) in response to the treatment of bacterial suspension (C1L) or culture filtrate (C1L CF) were analyzed different days after treatment with or without subsequent fungal inoculation (Be). The 0d, 1d, 4d, and 7d refers to days after *B. elliptica* inoculation and C1L alone treatment or after *B. elliptica* inoculation on C1L-treated plants. The 18S rRNA and 28S rRNA are the references for the amount of total RNA loaded.

were extensively suppressed when *B. elliptica* was inoculated on strain C1L-treated lily plants, we presumed the negative regulation is controlled by a signal transduction pathway induced by *B. cereus* strain C1L that differs from the pathogen-induced SA-directed pathway.

ACKNOWLEDGMENTS

This study was supported by the Taroko National Park Headquarters, Ministry of the Interior, and the Council of Agriculture, Taiwan, Republic of China. We thank C.-H. Lin and Y.-H. Yang for technical assistance and scientific discussion.

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