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Identification and transcriptional analysis of genes involved in *Bacillus cereus*-induced systemic resistance in *Lilium*

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Abstract

Bacillus cereus C1L was demonstrated to induce systemic resistance in *Lilium formosanum* against leaf blight caused by *Botrytis elliptica*. Suppression subtractive hybridization library of *L. formosanum* triggered by *B. cereus* C1L were screened and 3 differentially expressed genes were identified. Based on sequence analysis, these genes encoding putative glycine-rich protein, metallothionein-like protein, and PsbR protein of photosystem 2, were designated *LfGRP1*, *LfMT1*, and *LfPsbR*, respectively. The results of Northern blot analysis showed that expressions of *LfGRP1*, *LfMT1* and *LfPsbR* increased in response to *B. elliptica* infection. On the other hand, expression of *LfMT1* increased but expressions of *LfGRP1* and *LfPsbR* decreased when the rhizosphere of *L. formosanum* was drenched with suspension of *B. cereus* C1L with or without subsequent challenge with *B. elliptica* on lily leaves. Similar expression profiles of homologues of *LfGRP1*, *LfMT1*, and *LfPsbR* (named *LsGRP1*, *LsMT1*, and *LsPsbR*, respectively) were presented in *Lilium* oriental hybrid Star Gazer. In addition, application of the photosynthetic inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, on the leaves reduced disease severity and expressions of *LsGRP1* and *LsPsbR* just as that in response to *B. cereus* C1L treatment.

Additional key words: lily, differential gene expression, defense-related genes, DCMU.

Introduction

Botrytis leaf blight caused by the fungal pathogen, *Botrytis elliptica* (Berk.) Cooke, is one of the most important diseases of lily (Brooks 1980, MacDaniels and Horst 1980). For the control of Botrytis leaf blight of lily, *Bacillus amyloliquefaciens* B190 (Chiou and Wu 2001, 2003) and *Bacillus cereus* 28-9 (Huang *et al*. 2005) have been shown to be potential biocontrol agents. Systemic acquired resistance (SAR) is a whole-plant resistance that occurs following an earlier localized exposure to a pathogen and the SAR response can be induced chemically (Kessmann *et al*. 1994, Durrant and Dong 2004). In addition, systemic resistance of plants against pathogen attack can be induced by non-pathogenic microorganism, and is known as induced systemic resistance (ISR; Van Loon *et al.* 1998, Zehnder *et al*. 2001, Kloepper *et al.* 2004, Bakker *et al.* 2007). In lily, application of a rhizobacterium *Bacillus cereus* C1L to the plant rhizosphere was demonstrated as a good strategy to protect the leaves from *B. elliptica* attack (Liu *et al*. 2008).

SAR and ISR are regulated by different signaling pathways to activate resistance-associated gene expression and defense response (Van Loon *et al*. 1998, Durrant and Dong 2004). To dissect signaling pathways and gene expressions of plants responsible for systemic resistance, molecular analyses of SAR and ISR mechanisms have been performed in *Arabidopsis* but less information has been obtained from other plant systems. In our laboratory, we focus on systemic resistance of lily against Botrytis leaf blight. In previous studies, the phenomenon of SA-induced systemic resistance of *Lilium* oriental hybrid Star Gazer against *B. elliptica* infection was investigated and a differentially expressed gene *LsGRP1* encoding a putative glycine-rich protein was identified (Chen and Huang 1997, Lu and Chen 1998, 2005). In addition, increased *LsGRP1* expressions in response to *B. elliptica*, probenazole, and salicylic acid (SA) were demonstrated (Lu and Chen 2005, Lu *et al*. 2007). Since the rhizobacterium *B. cereus* C1L-tirggered systemic resistance was manifested in lily (Liu *et al*. 2008), the driving mechanism became an intriguing subject in our investigations.

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Abbreviations: SSH - suppression subtractive hybridization; DCMU - 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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Materials and methods

Bacillus cereus C1L was cultured in Luria-Bertani (LB) broth (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl) at 28 °C with shaking for 16 h. Cells of strain C1L were harvested by centrifugation (4 000 *g* for 10 min) and resuspended in 10 mM $MgSO_4$ to a final concentration of 1×10^8 cfu cm^{-3} for the drench application to the rhizosphere of lily plants. *Escherichia coli* XL1-Blue (*Stratagene*, La Jolla, CA, USA) was used as a host for gene cloning. All bacterial strains were cultured on LB agar plate (LB broth with 1.5 % agar). Bacterial strains were suspended in 15 % glycerol for long-term storage at -80 °C. *B. elliptica* B061 was cultured on V-8 agar plate for sporulation (Huang *et al*. 2005). A conidial suspension of strain B061 was prepared in 0.05 % *Tween-20* and adjusted to a final concentration of 5×10^4 conidia cm^{-3} for inoculation on lily leaves.

Seedlings of *Lilium formosanum* Wall., a native species of lily in Taiwan, and the bulbs of *Lilium* oriental hybrid Star Gazer were planted in potting mix (peat moss and *Perlite* at a ratio of 3:1) and grew at temperature of 20 - 23 °C, 12-h photoperiod, irradiance of 100 μmol m^{-2} s^{-1} and air humidity of 80 %. In this study, 30 to 45-d-old plants were used. *L. formosanum* was mainly used in the construction of suppression subtractive hybridization (SSH) library and the analysis of gene expression by Northern blot hybridization was performed in both *L. formosanum* and *Lilium* Star Gazer.

Three days before RNA extraction from lily leaves, the rhizosphere of *L. formosanum* plant was drenched with 50 cm^3 of bacterial suspension of strain C1L as a tester. For preparation of mRNA as a driver, the rhizosphere of *L. formosanum* plant was applied with equivalent amount of 10 mM $MgSO_4$ in place of bacterial suspension. For each treatment, total RNA was extracted from middle leaves according to the method descried by Lu and Chen (2005) and the mRNA was further purified using the *Oligotex* direct mRNA kit (*Qiagen*, Hilden, German). SSH was performed using a PCR-select cDNA subtraction kit (*Clontech*, Palo Alto, CA, USA) and the product was cloned into pGEMT-easy vector (*Promega*, Madison, WI, USA). SSH was used to selectively amplify target cDNA fragments (differentially expressed) and simultaneously suppress nontarget DNA amplification, based on the suppression PCR effect.

The inserts of individual clones from the SSH library were amplified by colony PCR for dot blot analysis. The denatured PCR products (1 mm^3) were dot-blotted onto *Hybond-N*$^+$ nylon membranes (*GE Biosciences*, Waukesha, WI, USA). Dot-blotting was done on two membranes for hybridization with two different DIG-labeled cDNA probes. One membrane was hybridized with probe prepared from the mRNAs isolated from the leaves of *B. cereus* C1L-treated *L. formosanum* and the other was hybridized with that of $MgSO_4$-treated *L. formosanum*. After screening, clones showing different hybridization intensities were selected for nucleotide sequencing. Sequence analysis was performed using the software from National Center for Biotechnology Information (NCBI) and *Vector NTI* advance software version *8.0* (*Invitrogen*, Carlsbad, CA, USA).

Total RNA was isolated from samples representing each treatment and the control according to the method descried by Lu and Chen (2005). For RNA gel blot analysis, 10 μg of total RNAs were separated on a denaturing 1.0 % formaldehyde agarose gel and subsequently transferred onto a *Hybond-N*$^+$ nylon membrane; the membrane was probed with a DIG-labeled DNA probe, washed, and subjected to detection using the *DIG* luminescent detection kit (*Roche Diagnostics*, Mannheim, Germany). The DNA probes were prepared as partial cDNA fragments amplified from cDNA clones using the PCR DIG probe synthesis kit (*Roche Diagnostics*).

To investigate the involvement of photosynthetic system in the systemic resistance induced by *B. cereus*, a photosynthetic electron transfer inhibitor, DCMU, at concentrations of 25, 50 and 100 μM were sprayed on the expanding leaves of lily plants before fungal inoculation. Sterile deionized water was used as a control. At 24 h after DCMU treatment, a conidial suspension of *B. elliptica* was atomized on the leaves and the inoculated plants were incubated at 20°C and 100 % humidity. The number and size of lesions were recorded 3 d after fungal inoculation. Each treatment had 5 plants and 3 leaves in each plant were inoculated. In addition, total RNA of the leaves were extracted for RNA blot analysis.

The nucleotide sequence of *LfMT1* and *LfPsbR* have been deposited in the GenBank database under accession number EU371024 and EU371025.

Results

L. formosanum, which is highly susceptible to *Botrytis* leaf blight, developed severe symptom after *B. elliptica* infection, when the plant was treated only with 10 mM $MgSO_4$. However, symptom development on the leaves was significantly reduced when *L. formosanum* was drenched with bacterial suspension of strain C1L 3 d before inoculation with *B. elliptica*.

The cDNAs of *L. formosanum* associated with induction of *B. cereus* C1L were obtained after construction of SSH library and differential screening. The product of SSH was analyzed by gel electrophoresis and the cDNAs were observed as a smear of 200 to 650 bp without distinct bands (Fig. 1). In total, 193 individual clones were obtained and served as potential targets in differential screening by dot blot method. Most of the clones exhibited low signals and were hardly

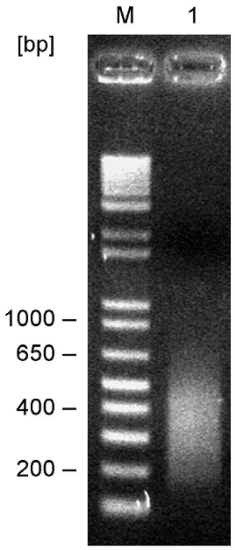

Fig. 1. Electrophoresis of cDNA products obtained from SSH. The rhizosphere of *L. formosanum* was drenched with bacterial suspension of strain C1L (50 cm^3) 3 d before RNA extraction and the middle leaves were used to extract mRNA as a tester. For preparation of mRNA as a driver, the rhizosphere of *L. formosanum* was drenched with equivalent amount of 10 mM $MgSO_4$ 3 d before RNA extraction. PCR-select cDNA subtraction product is shown in *lane* 1.

differentiated; anyhow, 8 clones exhibited distinct hybridization. Among them, 4 were up-regulated and 4 were down-regulated in response to strain C1L treatment.

The inserts of eight clones exhibiting different hybridization intensities were sequenced. The inserts of four up-regulated clones were derived from the cDNA that had sequence homologous to a gene encoding metallothionein-like protein of *Fritillaria agrestis***,** and the corresponding gene of lily was designated *LfMT1* (*Lilium formosanum* metallothionein-like protein 1). The putative peptide sequence of 63 amino acid residues contained 10 cysteines (16 %) and had a total of four Cys-X-Cys motifs (X = any amino acid, except Cys) in the N- and C-termini (Fig. 2). Among the insert sequences of four down-regulated clones, 3 were homologous to the glycine-rich protein-encoding gene *LsGRP1* (accession number AY072283) and had been reported by Liu *et al.* (2008) as *LfGRP1* (accession number EF681959). The other one was similar to a gene encoding PsbR protein of photosystem 2 of *Xerophyta humilis* and was designated *LfPsbR* (*Lilium formosanum* PsbR). Using the primers designed, *LsMT1* and *LsPsbR* were successfully amplified from *Lilium* Star Gazer. Nucleotide sequences of *LfMT1* and *LsMT1* or *LfPsbR* and *LsPsbR* shared 100 % identity, indicating that these genes are conserved in *L. formosanum* and *Lilium* Star Gazer.

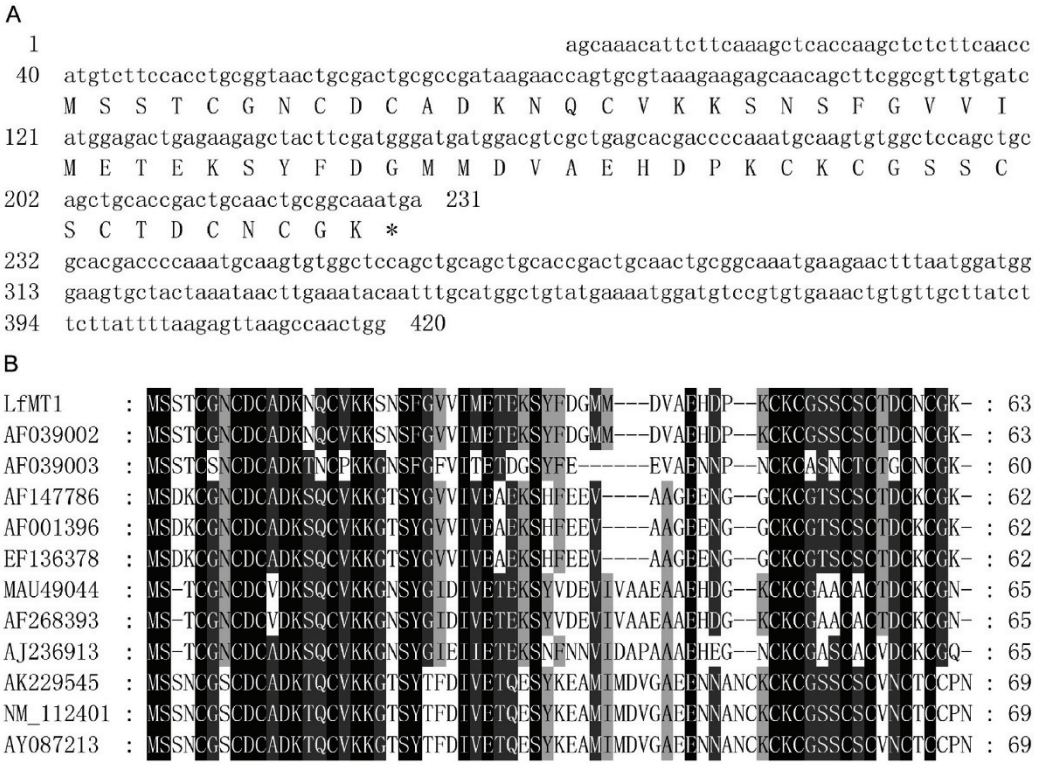

Fig. 2. Sequence analysis of *LfMT1*. *A* - The nucleotide and deduced amino acid sequences of *LfMT1*. *B* - Alignment of putative peptide sequences of LfMT1 and other MT-like proteins. LfMT1 is compared with MTs from *Fritillaria agrestis* (AF039002 and AF039003), *Oryza sativa* (AF147786, AF001396 and EF136378), *Musa acuminata* (MAU49044 and AF268393), *Elaeis guineensis* (AJ236913), and *Arabidopsis thaliana* (AK229545, NM_112401 and AY087213).

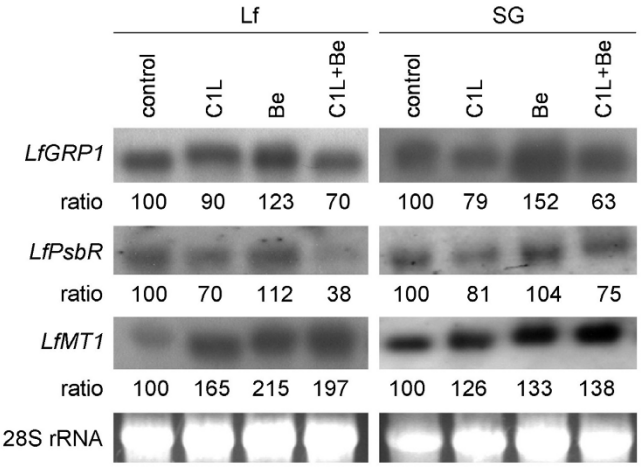

Fig. 3. Transcriptional expressions of *LfGRP1*, *LfPsbR*, *LfMT1* and their homologues in *Lilium*. Total RNAs (10 μg each sample) of *L. formosanum* (Lf) and *Lilium* Star Gazer (SG) were extracted after 3-d C1L treatment (C1L), 3 d after *B. elliptica* inoculation (Be) and *B. elliptica* inoculation (3 d) after 3-d C1L treatment (C1L+Be), and performed Northern blot analysis with *LfGRP1*, *LfMT1* and *LfPsbR* cDNA probes. The 28S rRNA is the reference for the amount of total RNA loaded. Quantitation of gene expression is indicated by a ratio.

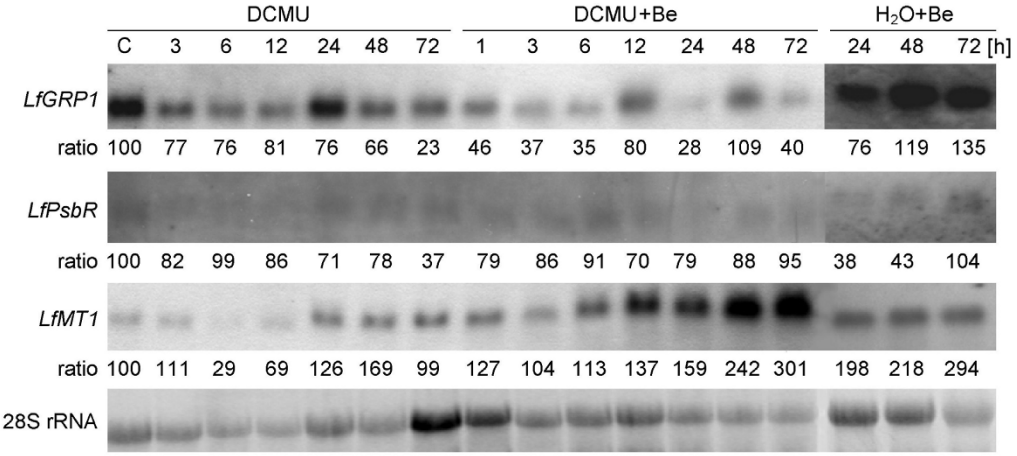

Fig. 4. Northern blot analysis of gene expression in the Star Gazer leaves after DCMU treatment. DCMU solution was applied to the adaxial surface (DCMU) and the leaves were subsequently inoculated with *B. elliptica* 24 h after DCMU treatment (DCMU+Be). For comparison, sterile deionized water was used instead of DCMU before inoculation with *B. elliptica* (H_2O+Be). Total RNAs of 10 μg per sample were loaded and Northern blot analyses were performed with *LfGRP1*, *LfPsbR* and *LfMT1* cDNA probes. The 28S rRNA is the reference for the amount of total RNA loaded. C - control. Quantitation of gene expression is indicated by a ratio.

The expressions of *LfGRP1*, *LfMT1*, and *LfPsbR* were investigated by Northern blot hybridization. When *L. formosanum* was treated with strain C1L 3 d before the extraction of RNA from the leaves, only *LfMT1* showed increased transcription, but *LfGRP1* and *LfPsbR* displayed slightly decreased transcription (Fig. 3). However, expressions of *LfGRP1*, *LfPsbR*, and *LfMT1* were all enhanced after fungal inoculation as compared to that of the control. However; when *L. formosanum* was inoculated with *B. elliptica* 3 d after drench application of strain C1L, the amount of *LfMT1* transcript increased but the contents of *LfGRP1* and *LfPsbR* transcripts decreased. This is similar to the results derived from strain C1L treatment alone. Since *B. cereus* C1L had been demonstrated to induce systemic resistance in *Lilium* Star Gazer (Liu *et al*. 2008), the expressions of *LsGRP1*, *LsPsbR*, and *LsMT1* in this hybrid were investigated. The results showed that the expression profiles of *LsGRP1*, *LsPsbR*, and *LsMT1* in Star Gazer were very similar to *LfGRP1*, *LfPsbR*, and *LfMT1* in *L. formosanum*.

To examine the involvement of negative regulation of *LfPsbR* gene expression in *B. cereus* C1L-induced resistance, a photosynthetic inhibitor DCMU was sprayed onto the adaxial surfaces of lily leaves 24 h before inoculation with *B. elliptica*. DCMU solutions (25, 50 and 100 μM) suppressed disease severity, and higher DCMU concentrations had better effects in protecting lily plants from infection by *B. elliptica*. Since DCMU at a concentration up to 100 μM did not inhibit conidial germination of *B. elliptica* (data not shown), resistance of

Lilium Star Glazer leaves to *B. elliptica* could be triggered by DCMU. Additionally, 50 μM DCMU was sprayed onto the adaxial surface of Star Glazer leaves and the gene expressions were analyzed by Northern blot hybridization. When the plants of *Lilium* Star Gazer were treated with DCMU or inoculated with *B. elliptica* 24 h after DCMU treatment, expression of *LsGRP1* and *LsPsbR* decreased. On the contrary, *LsMT1* displayed positive regulation of gene expression in both conditions. When lily plant was singly inoculated with *B. elliptica*, *LsGRP1*, *LsPsbR*, and *LsMT1* displayed increased expression (Fig. 4).

Discussion

In this study, defense-related genes, *LfGRP1* (down-regulated), *LfMT1* (up-regulated), and *LfPsbR* (down-regulated), of *L. formosanum* in response to *B. cereus* C1L were identified by using the techniques of SSH and differential screening. The expressions of *LfGRP1*, *LfMT1*, and *LfPsbR* were subsequently investigated by Northern blot hybridization. When *L. formosanum* was treated with strain C1L for 3 d, only *LfMT1* expression significantly increased but the expressions of *LfGRP1* and *LfPsbR* slightly decreased. This result coincided with the result of differential screening of the SSH library, indicating that construction of SSH library in this study was reliable.

The nucleotide sequences of *LfGRP1* and *LsGRP1* share 87 % identity and the expression profiles of the *LfGRP1* in *L. formosanum* and *LsGRP1* in *Lilium* Star Gazer are nearly identical. A previous study showed that high expression of *LsGRP1* in lily leaves could be induced by SA and *B. elliptica* (Lu and Chen 2005); on the contrary, *LsGRP1* and *LfGRP1* expression in lily leaves decreased when the roots of lily plants were treated with strain C1L with or without subsequent challenge with *B. elliptica*. Thus, we suggest that *LsGRP1* and *LfGRP1* are differently regulated and play different roles in the SAR and ISR in lily. As reported by Chen *et al.* (1996), *B. cereus* UW85 did not induce SAR-related gene expression of tobacco seedlings. When the roots of tobacco seedlings were treated with *B. cereus* UW85, accumulation of *PR-1a* (SAR-related gene) transcript in the seedlings was not detected by Northern blot hybridization.

Metallothioneins (MTs) are small polypeptides with high percentage of cysteine residue, which may play an important role in metal detoxification and homeostasis (Hamer 1986, Robinson *et al*. 1993). Plants increase metallothionein (MT) expression as part of the defense mechanism in response to high contents of metals (Hsieh *et al*. 1995, Zhou and Goldsbrough 1995). In addition, expression of plant MT-like genes can be induced by other abiotic and biotic factors, including air pollution (Etscheid *et al*. 1999), cold storage (Reid and Ross 1997), drought (Akashi *et al*. 2004), ethylene (Coupe *et al*. 1995, Liu *et al*. 2002), heat shock (Hsieh *et al*. 1995), senescence (Buchanan-Wollaston 1994), wounding (Kim *et al*. 2001), fungal infection (Kim *et al*. 2001), and virus infection (Choi *et al*. 1996). Nevertheless, rhizobacterium-induced expression of MT-like gene has not been reported yet. In this study, a rhizobacterium-induced novel MT-like gene (*LfMT1*) was identified in *L. formosanum* for the first time. Expression of *LfMT1* was induced not only by the rhizobacterium *B. cereus* C1L but also by the fungal pathogen *B. elliptica*. Furthermore, *LfMT1* was highly expressed in *B. cereus* C1L-treated *L. formosanum* plants that were subsequently challenged by *B. elliptica*. This expression profile of MT-like gene has not been reported previously. Although functions of MT-like polypeptides in plant resistance to pathogen infection are not clarified yet, we tend to suggest the involvement of LfMT1 and its homologues in the disease resistance of lily. Akashi *et al.* (2004) identified a CLMT2 from wild watermelon that had high scavenging activity in the toxification of hydroxyl radicals and CLMT2 effectively suppressed hydroxyl radical-catalyzed degradation of watermelon genomic DNA (Akashi *et al*. 2004). In the study of Van Baarlen *et al*. (2004), accumulations of hydrogen peroxide and fragmented genomic DNA were observed in lily leaves after infiltration of *B. elliptica* culture filtrate. Therefore, we suggest that *LfMT1* expression induced by *B. elliptica* is a defense mechanism of *L. formosanum* to suppress the toxicity of ROS on genomic DNA. The high expression of *LfMT1* in response to *B. cereus* C1L treatment and *B. elliptica* infection may represent a general response to the interactions with pathogenic and non-pathogenic microbes.

According to the previous studies, PsbR is a 10 kDa extrinsic protein of the photosystem 2 (PS 2) complex and probably has two distinct roles in the function of the PS 2 complex (Suorsa *et al*. 2006, Allahverdiyeva *et al*. 2007, De Las Rivas *et al*. 2007). PsbR clearly stabilizes the PS 2 complex affecting the properties of electron transfer reactions in both acceptor and donor sides (Allahverdiyeva *et al*. 2007, De Las Rivas *et al*. 2007). Moreover, PsbR is crucial in providing additional ion concentration for the function of water oxidizing complex, which is likely to occur by stabilizing the binding of PsbP and PsbO proteins in the PS 2 complex (Suorsa *et al*. 2006). However, involvement of PsbR in plant resistance to pathogen infection has not been investigated yet. Based on the expression pattern of *PsbR*-like gene in *L. formosanum* and *Lilium* Star Gazer, we presume that PsbR possibly participates in plant resistance to a necrotrophic fungal pathogen as *B. elliptica*.

In addition, our results showed that DCMU had a notable effect on lily to suppress *B. elliptica* infection and *LsPsbR* displayed a negative regulation of gene expression. When *Pseudomonas thivervalensis* MLG45 was applied to the rhizosphere of *Arabidopisis*, the expression of chloroplast-related genes were also under negative regulation (Cartieux *et al*. 2003). Besides, oxygen evolving enhancer protein 2, an important component of

photosystem 2, was down- regulated in thaumatin-like protein overexpressed transgenic rice that exhibited enhanced resistance against bacterial blight (Mahmood and Komatsu 2009). Thus, we presume that the chloroplast plays an important role in the disease resistance of lily as induced by *B. cereus*.

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