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Stomatal closure, callose deposition, and increase of LsGRP1-corresponding transcript in probenazole-induced resistance against *Botrytis elliptica* in lily

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Abstract

Botrytis leaf blight caused by *Botrytis elliptica* (Berk.) Cooke, causes enormous loss in the cut-flower industry of lilies in Taiwan. Application of probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide) before fungal inoculation was shown to be effective in protecting Oriental lily cultivar Star Gazer from infection by *B. elliptica*. The protection occurred 1 day after probenazole treatment, achieved a significant level second day after treatment and was maintained at a high level for 14 days. Alterations in the pathogenic fungus and the host plant caused by probenazole treatment were examined. Probenazole treatment caused a reduction of conidial germination of *B. elliptica* to a lesser extent but a significant decrease of the fungal penetration rate. On the plant, a high ratio of foliar stomata appeared closed as induced by probenazole and such high ratio of closed stomata was maintained after inoculation with *B. elliptica*. As indicated by the effect of abscisic acid, stomatal closure would be one of the plant responses related to the reduction of *B. elliptica* infection by probenazole. A complex response can be induced following probenazole treatment in lily and the correlations with callose deposition and increase of *LsGRP1*-corresponding transcript are indicated.

Keywords: Lilium; Botrytis elliptica; Probenazole; Conidial germination; Penetration; Stomatal closure; β-1,3-Glucan polymer; LsGRP1; Abscisic acid

1. Introduction

Botrytis leaf and blossom blight caused by *Botrytis elliptica* (Berk.) Cooke, causes severe loss in the cut-flower production of lilies (*Lilium* spp.) in Taiwan [1]. Fungicide-resistant strains of *B. elliptica* have frequently evolved in the field so that the effectiveness of chemical control is substantially diminished after several years of use [2]. Alternative control measures have been developed in different aspects, such as biological control by beneficial microorganisms, application of film-forming polymers and induction of host resistance by plant activators, salicylic acid (SA) and probenazole [3–7]. Probenazole (3-allyloxy-1,2-benzisothiazole-1, 1–dioxide), an active ingredient of Oryzamate[®], is capable of suppressing disease development in many plants, as demonstrated in rice, tobacco, *Arabidopsis* and lily [6–12]. Probenazole affecting infection

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process of fungal pathogen has been reported in rice blast disease. Conidial germination of Magnarporthe grisea and the penetration of rice plants were retarded when the plants were treated with probenazole [9–11]. Alterations of plant tissues after probenazole treatment may exhibit in different features. Stomatal closure has been observed in lily leaves as induced by SA [7]. Callose deposition, an increase of β -1,3-glucan polymer, in the guard cells of foliar epidermis is another alteration of lily plants in response to SA [7]. Best known for probenazole-induced responses in plants are the increases of expression of defense-related genes such as PR-1 in Arabidopsis, PR-1, PR-2 and PR-5 in tobacco, PBZ1 and RPR1 in rice [8,12–18]. A cDNA named LsGRP1, encoding a putative glycine-rich protein, has been cloned from Oriental lily cv. Star Gazer in our laboratory. Increase of LsGRP1corresponding transcript is correlated to the SA-induced resistance against B. elliptica in lily [7]. In this study, we examined the induction and duration of probenazole-induced resistance in lily toward Botrytis leaf blight and the infection process of B. elliptica as affected by probenazole. To better understand the defense mechanism driven by probenazole in

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lily, the status of stomata, callose deposition, and the *LsGRP1* gene expression were investigated. In addition, the relatedness of the status of stomata to the suppression of *B. elliptica* infection was further examined on the responses of lily leaves to abscisic acid treatment.

2. Materials and methods

2.1. Lily planting and preparation of fungal inoculum

The bulbs of *Lilium* oriental hybrid cv. Star Gazer were planted in 14-cm pots (one bulb per pot) containing potting mix of a commercial medium (Bas Van Burren, Maasland, the Netherlands) and perlite at a ratio of 3:1 and grown in a growth room at 20–23 °C with light/dark cycles of 12 h each for 30–40 days. The sporulation culture of *B. elliptica* B061-1 was grown on V-8 agar (20% V-8 vegetable juice (Campbell soup group), 0.3% CaCO₃, 1.5% agar) under near UV light for 5 days [7,19,20]. The conidia were collected in Tween 20 solution (0.05% Tween 20 in sterile deionized water) from fungal culture by gentle vortexing.

2.2. Disease suppression assay

Probenazole (6% active ingredient in Oryzamate[®] purchased from Taiwan San Lee Chemical LTD.) was directly applied to the rhizosphere of 'Star Gazer' plants in a concentration of 40 mg a.i. kg⁻¹ potting mix. At different periods after application of probenazole, the abaxial surface of lily leaves was atomized with *B. elliptica* conidial suspension of 5×10^4 conidia ml⁻¹. Five plants were used for each treatment. Three middle leaves were inoculated. Symptom development was examined 3 days after fungal inoculation and compared to that without probenazole treatment. Data were subjected to analysis of variance (ANOVA) and the least significance difference test.

2.3. Examination of the initial infection process of B. elliptica on lily leaves

To microscopically examine the effect of probenazole treatment on B. elliptica-infection process, a detached leaf assay was performed. Firstly, probenazole was applied to the rhizosphere of 'Star Gazer' plants. After probenazole treatment, fully expanded leaves from middle portion of lily plants were detached and placed in moist petri dishes. The cut ends of petioles were immersed in sterile water. Meanwhile, the 'Star Gazer' plants without probenazole treatment were used as a control. For examination on the conidial germination of B. elliptica on lily leaves, aliquots (10 µl) of B. elliptica conidial suspension containing 5×10^4 conidia ml⁻¹ were pipetted onto the abaxial surface of detached leaf. After incubation for 12 h, conidial germinations in the inoculation fluids were examined under a microscope. Conidial germination rate was measured according to the data from three inoculation fluids and the experiment was repeated twice. For examination on the penetration of lily leaves by B. elliptica, aliquots (10 µl) of conidial suspension containing 5×10^2 conidia ml⁻¹ were pipetted onto the abaxial surface of detached lily leaves with or without probenazole treatment (10 drops each leaf). Different periods after inoculation, lily leaves were subjected to clearing process with 95% ethanol and further with 1% SDS treatment. Subsequently, the leaves were stained with 0.1% Coomassie blue in 40% ethanol and 10% acetic acid [21]. After proper de-staining, the leaves were examined under a light microscope (Leica DMR, Wetzlar, Germany) for the frequency of penetration by *B. elliptica*. Three leaves were used for each time period and the experiment was repeated twice.

2.4. Examination of the alterations of epidermis of lily leaves

The alterations of foliar epidermis of lily plants caused by probenazole treatment were examined microscopically and compared with those without probenazole treatment. The status of stomata and callose deposition were investigated. The middle leaves were detached from lily plants and placed in moist petri dishes. Aliquots (10 µl) of B. elliptica conidial suspension containing 5×10^4 conidia ml⁻¹ were pipetted onto the abaxial surface of detached leaves. Tween 20 solution was used instead of conidial suspension as a control. Different periods after fungal or mock inoculation, the leaves were subjected to clearing with 1 M KOH solution at 121 °C for 15 min and followed by aniline blue staining (0.05% aniline blue dye WS (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA) in 0.067 M K₂HPO₄, pH 9.0). The clearing and staining procedure followed that described in the previous report [7]. The status of stomata on 10 microscopical fields (at least 40 stomata each) was examined (Leica DM IL, Wetzlar, Germany) and the ratios of closed stomata were calculated. The experiment was repeated twice. The callose deposits were examined by epifluorescence illumination (340-380 nm excitation filter, 400 nm chromatic bean splitter, 425 nm barrier filter; Leica DM IL, Wetzlar, Germany) and the images were captured with a digital camera (COOLPIX990, Nikon, Japan).

2.5. Examination of the effect of abscisic acid

The effect of abscisic acid on the status of stomata and symptom development on lily leaves caused by *B. elliptica* were examined. Abscisic acid solution of 100 μ M was atomized onto the abaxial surface of lily leaves before atomization with conidial suspension of 5×10^4 conidia ml⁻¹. The procedures of sample preparations for the observation of the status of stomata and the measurement of the rate of stomatal closure followed that were described above. On the other hand, symptom development was examined 3 days after fungal inoculation. Five plants were used for each treatment. Three middle leaves were inoculated in a plant. Data were subjected to analysis of variance (ANOVA) and the least significance difference test. These experiments were repeated twice.

2.6. RNA blot analysis

The leaves of lily plants treated with probenazole were inoculated with *B. elliptica* conidial suspension or mock

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inoculated. Different periods after inoculation, the leaves were detached and stored at -80 °C until extraction of RNA. Total RNA of lily leaves was isolated using a Plant Total RNA Extraction Miniprep System (Viogene, CA, USA) and subjected to electrophoresis on 1% agarose gel which was prepared in 2.2 M formaldehyde-containing $1 \times$ MOPS buffer (40 mM MOPS, pH 7.0, 10 mM sodium acetate, 1 mM EDTA), using $1 \times$ MOPS running buffer. The gel was transferred to a positively charged HybondTM-N⁺ nylon membrane (Amersham Biosciences) by capillary transfer method. The membrane was UV cross-linked and hybridized with the DIG-labelled LsGRP1 probe prepared by using PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The SA-inducible LsGRP1 cDNA, encoding a putative glycine-rich protein, has been isolated from lilv cv. Star Gazer in our lab [7]. After hybridization, the membrane was processed following the protocol of DIG Luminescent Detection Kit for nucleic acids (Roche Diagnostics, Mannheim, Germany). At the beginning, membrane was washed with washing buffer (100 mM maleic acid, 150 mM NaCl, 0.3% Tween 20, pH 7.5) for 5 min at room temperature. Afterwards, the membrane was incubated in a blocking solution (1% blocking reagent in maleic buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5)) for 30 min and further in an antibody solution (1:10000 dilution of Anti-Digoxigenin-AP (Roche Diagnostics, Mannheim, Germany) in blocking solution) for 30 min. Following two successive rinses in washing buffer, the membrane was incubated with 1% chemiluminescent substrate CDPstarTM (Tropix Inc., Bedford, MA, USA) in a buffer (100 mM Tris-HCl, 100 mM NaCl, and 50 mM MgCl₂) for 10 min. The chemiluminescent signals generated were recorded by exposure to HyperfilmTM ECL films (Amersham Biosciences).

3. Results

3.1. Disease suppression by the treatment with probenazole

To test the time period required for disease suppression, probenazole was applied to the rhizosphere of lily plants at different times before inoculation with *B. elliptica*. Low level of protection was attained 1 day after treatment; however, the number of lesions significantly decreased 2 days after treatment. A 70% to 85% disease suppression was observed as compared to the control without probenazole treatment. This suppression could last for 14 days after probenazole treatment (Table 1).

3.2. Conidial germination on the foliar surface of lily plants treated with probenazole

Conidial germination of *B. elliptica* on the foliar surface of lily plants was examined by the detached leaf method. Probenazole treatments were done 1 and/or 4 day(s) before fungal inoculation. The conidial germinations in different treatments were compared at 12 h after inoculation. It appeared that conidial germination on the foliar surface of lily plants was inhibited 15–25% by probenazole treatment at 1 or 4 day(s)

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Table	1	

Effect of probenazole treatment on lesion development caused by B. elliptica

Day(s) after probenazole treatment ^a	Number of lesions per leaf ^b			
1	$9.18 \pm 1.35^{ m c}$			
2	$3.24 \pm 1.78^{*d}$			
3	$3.64 \pm 1.60^{*}$			
7	$1.82\pm0.98^*$			
14	$2.06\pm0.13^*$			
Control	12.36 ± 5.48			

^a Probenazole was applied to the rhizosphere of 'Star Gazer' plants before fungal inoculation.

^b Conidial suspension of *B. elliptica* was atomized on the abaxial surface of lily leaves after probenazole treatment. Number of lesions per leaf was recorded 3 days after fungal inoculation.

^c Value = mean \pm standard deviation (five replications).

^d *Significantly less than the control (P = 0.05).

before fungal infection. The double treatment within a week did not further increase the inhibition rate of conidial germination (Fig. 1).

3.3. Effect of probenazole treatment on penetration of lily leaves by B. elliptica

In our observation, a large portion of inoculated conidia of *B. elliptica* on 'Star Gazer' leaves could germinate and penetrate the foliar epidermis directly or through the stomata. The penetration of lily leaves by *B. elliptica* conidia occurred 12 h after fungal inoculation and over 80% of the deposited conidia penetrated into the foliar surface. However, when the lily plants were treated with probenazole 4 days before fungal inoculation, 70% inhibition on the penetration by *B. elliptica* was achieved. The low level of penetration rate was maintained up to 72 h after fungal inoculation (Fig. 2).



Fig. 1. Effect of probenazole on conidial germination of *B. elliptica* on the lily leaves. The germination rate of conidia on foliar surface was recorded 12 h after inoculation. PBZ1d and PBZ4d represent fungal inoculation 1 or 4 day(s) after probenazole treatment, respectively; PBZ1 + 4d represents fungal inoculation after twice probenazole treatment. CK, fungal inoculation on lily leaves without probenazole treatment. Values are shown as the average with standard deviation.



Fig. 2. Penetration of lily leaves by *B. elliptica* affected by probenazole treatment. *B. elliptica* conidia were inoculated on lily leaves 1 day after probenazole treatment. Penetration of lily leaves was examined by light microscopy after clearing and staining procedures. Penetration rates were calculated from 50 conidia per leaf with three replications on each time point after fungal inoculation. Values are shown as the average with standard deviation.

3.4. Stomatal closure in response to probenazole treatment

To find out whether the treatment of probenazole would affect the response of foliar epidermis as the SA treatment did [7], the stomata of the foliar surface of lily plants with probenazole treatment were examined by light microscopy after clearing process of the leaves. The results showed that probenazole could cause stomatal closure as early as 1 day after treatment and the ratios of closed stomata were maintained at high level after subsequent fungal inoculation (Fig. 3). Many stomata on the *B. elliptica*-inoculated leaves appeared closed regardless of whether the treatment of probenazole was done 1, 4 or 7 day(s) before fungal inoculation. In contrast, many stomata were kept opened on the untreated leaves after fungal inoculation as that with mock inoculation.



Fig. 3. Stomatal closure of lily leaves in response to probenazole treatment and *B. elliptica* infection. *B. elliptica* was inoculated on lily leaves 1 or 4 day(s) after probenazole treatment. Tween 20 solution (Tw) was used instead of conidial suspension as mock inoculation. Different periods after inoculation, the leaves were subjected to clearing procedure and examined on the status of stomata by light microscopy. Values are shown as the average with standard deviation.



Fig. 4. Stomatal closure on lily leaves induced by abscisic acid. The abaxial surface of lily leaves was atomized with 100 μ M abscisic acid (ABA) prior to clearing process for examination on the status of stomata by light microscopy. Especially, the status of stomata was examined on ABA-treated (3 days) leaves after inoculation with *B. elliptica* (ABA + Be). Values are shown as the average with standard deviation.

3.5. Effect of abscisic acid

In order to clarify the correlation of stomatal closure to probenazole-induced resistance in lily, a solution containing 100 μ M ABA was atomized onto the abaxial surface of lily leaves before fungal inoculation and the status of stomata and symptom development were examined. A high ratio of closed stomata appeared as early as 6 h after ABA application and lasted for 7 days (Fig. 4). When the lily leaves were inoculated with *B. elliptica* 3 days after ABA treatment, the ratio of closed stomata decreased to a lower range; however, the number of lesion developed on lily leaves could reduce to one half of that on the untreated leaves. A less degree of reduction in the number of lesions was achieved when fungal inoculation was performed 1 or 2 day(s) after ABA treatment (Table 2).

3.6. Appearance of fluorescence in the guard cells of lily leaf epidermis as shown by aniline blue staining

In order to know whether probenazole could cause deposition of β -1,3-glucan polymer as that observed in SA-treated lily plants [7], lily leaves processed through aniline blue staining procedure were examined by fluorescence microscopy.

Table 2						
Effect of abscisic aicd	(ABA) on	lesion	development	caused	by <i>B</i> .	elliptic

Day(s) after ABA treatment ^a	Number of lesions per leaft		
1	$17.20 \pm 3.67^{\circ}$		
2	17.13 ± 0.99		
3	$12.40 \pm 2.60^{*d}$		
Control	21.20 ± 2.62		

 a The abaxial surface of lily leaves was atomized with 100 μM ABA before fungal inoculation.

^b Conidial suspension of *B. elliptica* was atomized onto the abaxial surface of lily leaves after ABA treatment. Number of lesions per leaf was recorded 3 days after fungal inoculation.

^c Value = mean \pm standard deviation.

^d *Significantly less than the control (P = 0.05).

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In the untreated condition, the guard cells of lily leaves did not show fluorescence at 6 h or 24 h after *B. elliptica* infection, as that shown in the mock inoculated control leaves (Fig. 5A and B). When lily plants were treated with probenazole 4 days before fungal inoculation, many guard cells appeared fluorescent 24 h after fungal inoculation; fluorescence was also observed in the guard cells with mock inoculation but in a lesser degree (Fig. 5C and D). In addition, fluorescence frequently appeared in the anticlinal wall of epidermis near penetration sites on the inoculated leaves (Fig. 5E and F).

3.7. Gene expression of LsGRP1

To know whether the gene expression of lily leaves was altered by probenazole treatment, the *LsGRP1*-corresponding

transcript responsive to SA treatment was monitored. Total RNA of the leaves was analyzed at different times (12, 24, 48 h) after fungal inoculation on the leaves with 1 dayprobenazole treatment. The results showed an intensive increase of *LsGRP1*-corresponding transcript 12–48 h after fungal inoculation (Fig. 6, right panel). Whether probenazole singly could cause an alteration of defense gene expression of lily leaves was examined. The result showed that the *LsGRP1*-corresponding transcript increased in lily leaves 48 h after probenazole treatment and the signal was intensified with prolonged incubation, as shown in 72 h and 7 days after probenazole treatment (Fig. 6, left panel). Moreover, increase of *LsGRP1*-corresponding transcript was detected in lily leaves 10 and 15 days after probenazole treatment (data not shown).



Fig. 5. Callose deposition on the epidermis of lily leaves. The leaves from lily plants without (A, B) or with (C, D, E, F) 4-day probenazole treatment were mockinoculated (A, C) or inoculated with *B. elliptica* (B, D, E, F). After aniline blue staining, the leaves were examined by epifluorescence microscopy (A–D, F) or light microscopy (E). Fluorescence appeared in the guard cells and anticlinal wall of epidermis. The images were captured 24 h after inoculation. Bar = $50 \mu m$.



Fig. 6. Northern blot analysis of lily leaves. Total RNA was extracted from the leaves 12 h, 24 h, 48 h, 36 h, 72 h and 7 days (7d) after probenazole treatment, and the leaves 12 h, 24 h, and 48 h after *B. elliptica* (Be) inoculation on probenazole-treated plants. Probenazole 24 h, probenazole applied to the rhizosphere of lily plants 24 h before fungal inoculation. Total RNA of the leaves from untreated lily plants was used as a control (0 h). The signals on the blotted membrane detected by DIG-labeled *LsGRP1* probe is indicated by an arrow. The 18S rRNA and 28S rRNA are the references for the amount of total RNA loaded.

4. Discussion

Probenazole is capable of suppressing disease development in *Arabidopsis*, rice and tobacco [8–12] and has been used as a plant protection agent, mainly for the control of rice blast and bacterial blight [22–25]. We had demonstrated that probenazole can drive the prevention of *B. elliptica* infection on lily by regular application at 7-days intervals [6]. In this study, we showed that protection was significantly attained 2 days after application of probenazole and was maintained high level for 14 days. This protection is not only caused by an influence on the conidial germination of *B. elliptica* but also the retardation of the penetration of lily leaves by *B. elliptica*, indicating that the mechanism of induced disease resistance is likely involved in the plant protection by probenazole in lily.

This report is the first to describe the effect of probenazole on the status of foliar stomata of plants. A reduction of the penetration of rice by blast pathogen related to the probenazole treatment has been reported [9–11]. The accessory cells around the guard cells have been found to be the main penetration sites of *B. elliptica*; besides, penetration does occur through stomata [26]. According to our observations, stomatal closure would contribute in certain extent to the probenazole-induced defense response in lily against infection by *B. elliptica*. This relatedness was verified when abscisic acid was used as an inducer of stomatal closure in lily. The results indicated that high ratio of closed stomata induced by ABA would reduce successful infection of *B. elliptica* and the lesion development although the subsequent fungal infection could cause an increase of the ratio of opened stomata.

Other defense responses would be involved in probenazoleinduced resistance against *B. elliptica*, as shown by our succeeding examinations on callose deposition and the *LsGRP1* gene expression. The results indicate that a combination of defense responses induced by probenazole is involved in the suppression Botrytis leaf blight in lily. Aniline blue has been used to detect amorphous β -1,3-glucan polymer (callose) constituent of plants [27–30]. It is known that callose deposition during the attempted infection by fungi is one of the defense responses of plants [29,31]. The presence of fluorescence in the guard cells and the nearby anticlinal cell walls of *B. elliptica*-inoculated lily leaves following the elicitation by both plant activators, SA [7] and probenazole (this study) implicate that callose deposition is involved in the defense of lily against fungal attack.

Probenazole has been known to induce gene expression in Arabidopsis, rice and tobacco [8,12–18]. Our laboratory has cloned a SA-inducible LsGRP1 from lily, which displays inducibility in response to SA and B. elliptica [3,7]. By the results of this study, LsGRP1 involvement in probenazoleactivated defense response against fungal attack is presumed. The fact that the expression of *LsGRP1* apparently increased 48 h after probenazole treatment and equivalent level maintained for 15 days is coincided with the period of protection by probenazole in lily. LsGRP1 is possibly involved in plant cell wall structural enforcement or signal transduction as that reported for the role of AtGRP3 in Arabidopsis [32-35]. Since callose deposition and increase of LsGRP1-corresponding transcript concurred in the leaves of probenazole- and SAtreated lily plants, the involvement of glycine-rich protein in callose deposition as addressed in tobacco system [36] may occur in lily and requires further investigation.

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