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

Yu-Yen Lu [1], Yi-Hung Liu [1], Chao-Ying Chen *

Department of Plant Pathology and Microbiology, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei 106, Taiwan, ROC

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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 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 *LsGRP1*-corresponding 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

* Corresponding author. Tel.: +886 2 33665207; fax: +886 2 23657735.
E-mail address: cychen@ntu.edu.tw (C.-Y. Chen).
[1] These authors contributed equally to this work.

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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% $CaCO_3$, 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 K_2HPO_4, 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 μM 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