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Molecular analysis of lily leaves in response to salicylic acid effective towards protection against *Botrytis elliptica*

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Abstract

The phytopathogenic fungus *Botrytis elliptica* (Berk.) Cooke causes necrosis and blight symptoms in lily leaves. Development of necrotic lesions in the leaves of Oriental lily cultivar Star Gazer could be suppressed by application of salicylic acid (SA) or previous infection by *B. elliptica*. In our observation, accumulation of β-1,3-glucan polymer in the guard cells and anticlinal walls of epidermis appeared more prominent in SA-treated leaves after *B. elliptica* infection as compared to that in SA-treated leaves with mock inoculation or in *B. elliptica*-inoculated leaves without SA treatment. Higher ratio of closed stomata appeared in SA-treated leaves after mock or *B. elliptica* inoculation, but less in *B. elliptica*-infected leaves without SA treatment. In molecular analysis of gene expression in response to SA treatment, a cDNA sequence coding for 138-amino acid protein was identified by suppression subtractive hybridization, followed by differential screening and 5′ RACE cDNA amplification. This putative protein designated LsGRP1 shared homology with several glycine-rich proteins present in plant extracellular matrix. Northern blot analysis detected an increase of *LsGRP1*-related mRNA transcript in 'Star Gazer' leaves after SA treatment, and also in *B. elliptica*-infected and upper un-inoculated leaves. When *B. elliptica* was inoculated on SA-treated leaves, accumulation of *LsGRP1*-related mRNA transcript appeared earlier as compared to that without SA treatment. That LsGRP1 or its homologues playing a role in SA and pathogen-induced defense responses in lily was thus presumed.
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Keywords: Lily; Salicylic acid; β-1,3-Glucan polymer; Stomatal closure; Glycine-rich protein; *Botrytis elliptica*

1. Introduction

Plants have evolved sophisticated preformed and induced defense mechanisms against pathogens and herbivorous insects. Localized infection by pathogens induces resistance to a wide range of pathogens such as fungi, bacteria or viruses, locally and systemically in the un-infected parts of the plant [1,2]. The defense mechanisms include various physical, physiological and biochemical alterations involving induction or potentiation of expression of a number of defense-related genes [1,3–6]. Salicylic acid (SA) is an endogenous signal for the activation of plant defense responses and essential for the expression of systemic acquired resistance (SAR) in many plants [7–10]. As known, exogenous application of SA induces plant resistance to different kinds of pathogens, that is associated with oxidative burst, cell wall enforcement, up- or down-regulation of gene expression, etc. [11,12].

Botrytis leaf and blossom blight, incited by *Botrytis elliptica* (Berk.) Cooke, causes severe losses in the cut-flower and bulb productions of lilies (*Lilium* spp.) in Taiwan [13]. Since this fungal infection occurs within a very short time under suitable conditions [13,14], frequent applications of fungicides are generally required to prevent prevalence of the disease. However, fungicide-resistant strains of *B. elliptica* have evolved frequently [15] and the efficacy of fungicide control of this disease is not always promising. During the last few years, application of plant activators to induce resistance becomes a new strategy for plant disease control [11,12,16] and is expected to be an alternative for effective and sustainable management of Botrytis blight in lilies [17]. Since multiple components are involved in the mechanisms of plant defense, the efficacy of induced

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resistance would last longer than that of protection by fungicide application. Understanding of plant defense responses would stimulate discovery of useful resources from plants and facilitate the development of effective disease control measures.

In this communication, we presented the effect of SA application in the control of Botrytis leaf blight in Oriental lily cultivar Star Gazer to show the potential of induced resistance in the protection of lily plants from fungal attack. The alterations of 'Star Gazer' leaves in response to SA treatment and *B. elliptica* infection were examined microscopically and analyzed molecularly to demonstrate the induced responses in this monocotyledonous plant. Microscopical examination mainly focused on the alterations of leaf epidermis, in particular the presence of β-1,3-glucan polymer and the stomatal opening. An attempt to clone defense-related genes induced by SA were conducted by suppression subtractive hybridization [18] and differential screening. By this approach, a SA-induced cDNA was identified from 'Star Gazer' leaves and used to demonstrate the inducibility of gene expression in response to SA and *B. elliptica*.

2. Materials and methods

2.1. Plant material and fungal culture

The bulbs of Oriental lily 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 12 h/12 h light/dark cycle for 15–30 days. *B. elliptica* strain 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 [19,20]. The conidia were collected from fungal culture by gently vortexing in Tween 20 solution (0.05% Tween 20 in sterile deionized water) and the conidial suspension was adjusted to a concentration of 5×10^4 conidia/ml.

2.2. SA treatment and pathogen inoculation for observation of induced resistance to B. elliptica

SA solution was freshly prepared by dissolving sodium salt of salicylic acid (Sigma Chemical Co., St. Louis, MO, USA) in deionized water. SA solution was drenched into the rhizosphere of each lily plant in a concentration of 0.4 mmol/kg potting mix before fungal inoculation. Conidial suspension of *B. elliptica* was atomized on the abaxial surface of middle leaves. Four leaves per plant were inoculated. Five plants were used for each treatment. In addition, for observation of SAR phenomenon, conidial suspension of *B. elliptica* was atomized on the upper leaves different days after first inoculation on the middle leaves of lily plants. Four leaves of each portion were inoculated. Lesion development was observed and recorded 3 days after inoculation. Normally, lily plants of 30 days old were inoculated with conidial suspension.

2.3. Histological examination of lily leaves

Fully expanded leaves in the middle portion of lily plants were detached and placed in Petri dishes for experiments. The cut end of the petioles was immersed in 0.1 mM SA solution or deionized water for different periods before microscopical examination. To investigate the effect of SA treatment on fungal attack, conidial suspension (6–8 drops per leaf, 10 μl each drop) was placed on the abaxial surface of lily leaves 24 h after SA treatment. Tween 20 solution was used for mock inoculation. For microscopical examination, lily leaves were processed through a procedure of aniline blue staining [20,21]. The leaves were immersed in 1 M KOH solution and autoclaved at 121 °C for 15 min, followed by three rinses with deionized water to remove KOH solution. Subsequently, the leaves were flooded with aniline blue dye (0.05% aniline blue WS (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA) in 0.067 M K_2HPO_4, pH 9.0) and examined by epifluorescence illumination (340–380 nm excitation filter, 400 nm chromatic bean splitter, 425 nm barrier filter; Leica DM IL, Wetzlar, Germany). The images were captured with a digital camera (COOLPIX990, Nikon, Japan). Aniline blue has been used to detect amorphous β-1, 3-glucan polymer constituent of fungi and plants [22,23]. In addition, stomata were examined and the ratios of closed stomata in microscopical fields were recorded. The data were collected from ten fields for each treatment.

2.4. Subtractive hybridization and PCR amplification

The rhizosphere of lily plants was drenched with SA solution in a concentration of 0.4 mmol/kg potting mix. The middle leaves were collected after SA treatment daily for three times and stored at −80 °C until pulverized in liquid nitrogen and extracted for RNA as a tester to screen differentially expressed mRNAs. Lily plants applied with equivalent amount of deionized water instead of SA solution were used to extract RNA as a driver. Total RNA was extracted from lily leaves using RNA extraction buffer (200 mM ammonium carbonate, 2% SDS, 2 mM disodium EDTA, 200 μg/ml bentonite, 1% 2-mercaptoethanol) followed by purification with acid phenol which was mixed with chloroform and isoamyl alcohol (24:1) at a ratio of 1:1, for at least three times. Two further RNA purification steps were conducted. In the first step, total RNA was precipitated by mixing with equal volume of 5 M lithium chloride overnight and collected by centrifugation at 4 °C for 20 min; subsequently, the RNA pellet was washed with 2 M lithium chloride and re-dissolved in RNase-free water. In the second step, total RNA was re-precipitated by mixing with 0.1 volume of 3 M sodium acetate (pH 4.8) and 2.5 volume of

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