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In vitro screening for *Botrytis* leaf blight resistance in *Lilium* species

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ABSTRACT

The plant genus *Lilium* is an economically important ornamental flowering monocot that is highly susceptible to leaf blight diseases caused by the fungus *Botrytis elliptica*. We designed an efficient *in vitro* screening system using a detached leaf disk assay to identify *Lilium* cultivars with resistance to *B. elliptica*. Optimal culturing conditions for the *B. elliptica in vitro* screening assay were established in which fungal isolates were grown on potato dextrose agar medium for 2 weeks at 25 °C under 12 h UV-A light. Healthy 2 weeks old *in vitro* grown leaf tissue from the susceptible cultivar 'Siberia' was inoculated with 2.0×10^3 spores·mL^{-1} *B. elliptica*, and incubated in the dark for 12 h at 25 °C. Seven days after inoculation, until blight symptoms on leaves with a significant disease index (DI) rate were observed. Using the standardized *in vitro* screening system with 'Siberia', we evaluated 30 different *Lilium* cultivars. The DI rates significantly varied among the different cultivars, suggesting that 30 cultivars could be classified into 3 groups: strong resistance (group A), moderate resistance (group B), and susceptible (group C). In order to elucidate the role of disease resistance (*R*)-genes in resistance/susceptible cultivars upon *Botrytis* infection, several well known marker *R*-genes were evaluated for their differential mRNA expression pattern through Real-time quantitative polymerase chain reaction (qRT-PCR). In general, qRT-PCR shows a remarkable up-regulated mRNA accumulation pattern of those genes in resistance (group A) as compared to that in susceptible (group C) cultivars, which further validating the accuracy of our *in vitro* screening system. This *in vitro* screening system is flexible and cost-effective, and will contribute to cultivar identification for *Botrytis* leaf blight in multiple *Lilium* cultivars.

1. Introduction

Lilium spp. is a perennial ornamental bulb plant species that has huge commercial value in the floriculture industry (Zhu et al., 2016). Lily, one of the three major flower crops, together with the chrysanthemum and rose, has become a major source of income for flower farming in Korea. The production of lily was about $28 million in 2012 on a 192 ha cultivation area (MAFRA, 2014), and their export has been increasing since the early 1990s, and in 2013, the export of lilies accounted for 32.5% of the total amount of exported cut flowers. The production of lily has been steadily increasing since the 1980s in the world, it was about on a 5542 ha cultivation area in 2006 (KREI, 2009).

Leaf blight in lilies is a serious threat to increased production in low-temperature and high-humidity environments mostly occurs during the humid (McRae, 1987; Doss et al., 1988; Hsieh and Tu, 1993). In the greenhouse, which is the main growth environment for lilies, blight symptoms occur not only on leaves but also on stems and petals. Also, the symptoms appear in the winter as well as summer, because proper temperature and humidity are always maintained for lily bulb and cut flower production in the greenhouse (Hsieh and Tu, 1993; Hou and Chen, 2003; Kim and Yun, 2014).

Leaf blight disease on lilies is caused by *Botrytis elliptica* and *Botrytis cinerea*, although *B. elliptica* is reported to be the major pathogen (KSPP, 2009). Disease symptoms caused by *B. elliptica* vary depending on the cultivar and vegetative stages of lily. Commonly, a brown oval lesion is formed on the petals and leaves, and severe symptoms include the formation of a large indeterminate lesion. At the late growth stage, the lesion turns pale yellow with a reddish or dark brown border and the infected leaves are withered. Leaf blight can be prevented by agronomical controls including crop rotation, rain-proof cultivation or by biological control using an antagonistic microorganism, but the prevention is mostly dependent on chemical control by germicide agrichemicals (Hahm et al., 2007). Currently, the usage of germicide agrichemicals is increasing with the expansion of lily cultivation (Lebiush-Mordechai et al., 2014); therefore, it is necessary to grow lily cultivars that have tolerance to leaf blight. Thus, a convenient and

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accurate screening method for evaluating resistance to leaf blight of lily cultivars is in high demanded.

The development of an *in vitro* test for resistance in lily cultivars using *B. elliptica* inoculation has not yet been reported. Hsieh and Huang (1999) have studied lily resistance to *B. elliptica* leaf blight using a film-forming polyelectrolyte and divided the calculated disease index (DI) into 0–4 categories according to leaf area infected. Additionally, Hsieh et al., (2001) reported on the infection process of *B. elliptica* inoculation on leaves of the oriental lily 'Stargazer'. Recent studies of *B. elliptica* and lily leaf blight are mostly focused on the lily defense reaction against *B. elliptica*, and reported the role of salicylic acid that is associated with systemically acquired resistance (Lu and Chen, 2005; Liu et al., 2008) and the physiological response to *Bacillus cereus* C1L-induced systemic resistance in lily (Huang et al., 2012).

RNA-seq technology has been a useful novel approach for the study of functional genomics in plants exposed to biotic and abiotic stresses at the transcriptome level (Liang et al., 2011). Digital gene expression profiling (DGE) is used to quantitatively study the genome-wide expression profiles of specific tissues in one species. DGE technology would enable us to identify numerous candidate genes of expressed sequence tags (EST) that are specifically or generally regulated at different stages of stress. The DGE technology has been used to compare disease resistance and susceptibility in infected tobacco plants using differential expression gene patterns (Lu et al., 2012). Studies have already identified differentially expressed genes and many biological processes involved in disease resistance responses in other plants like cotton or peony (Sun et al., 2013; Gong et al., 2015). These studies found that potential disease resistance-relevant genes could be effective against *Botrytis* infection in host plants; therefore, the application of transcriptome analysis in *Lilium* may identify potential *R*-genes and provide a useful tool in the study of the molecular mechanisms in the lily response to *Botrytis* infection. Previously, our research group built a cDNA library from the leaf tissue of *Lilium* lines followed by *Botrytis* infection (unpublished data, available under accession numbers; NN-3727-000001 and NN-3728-000001 at NABIC; http://nabic.rda.go.kr/). The current study utilized this transcriptomic resource to find several putative *R*-genes in *Lilium* by comparing the mRNA expression levels of candidate *R*-genes in healthy and infected plants.

In the present study, we developed a rapid and effective *in vitro* screening system to evaluate the quantitative differences between *Lilium* cultivars resistant and susceptible to leaf blight caused by *B. elliptica*. Based on a detached leaf assay, we screened 30 different *Lilium* cultivars in response to *B. elliptica* inoculation, of which 10 resistant and 6 susceptible cultivars were identified. The coordinated up- and down-regulation patterns of 8 *R*-genes in 3 selected resistance and susceptible cultivars further validated the accuracy of the *in vitro* screening method. Our results suggest that the *in vitro* screening system could be a reliable way to select and exploit *Lilium* cultivars resistant to *Botrytis* leaf blight disease.

2. Materials and methods

2.1. Plant material and in vitro cultures

The plant material used in this study consisted of 30 different *in vitro* grown lily cultivars of Asiatic lily ('Painted Pixie', 'Butter Pixie', 'Reinesse', 'Lavendel', 'Petit Pink', 'Disco', 'Black Out', 'Red Velvet', 'Pink Pagoda', 'Farfalla', 'Fantasy2′, and 'Connecticut King'), Longiflorum-Asiatic lily ('Suncrest', 'Royal Surprise'), Oriental lily ('Acapulco', 'Stargazer', 'ConAmore', 'Pesaro', 'Casablanca', 'Siberia', and 'Medusa'), Oriental-trumpet lily ('Anastasia' and 'Canca d'Or'), and Korean native lily {'Jejumal Nari' (*Lilium distichum*), 'Cham Nari' (*Lilium lancifolium*), 'Tdang Nari' (*Lilium callosum*), 'Summal Nari' (*Lilium hansonii*), 'Teoljoong Nari' (*Lilium amabile*), 'Nalgae Haneul Nari' (*Lilium dauricum*), and 'Daeman Nari' (*Lilium formosanum*)}. Additionally, the Oriental lily 'Siberia' was used as a control during the *in vitro* screening assays. The bulbs of all the lily cultivars were obtained from commercial growers in South Korea. For the *in vitro* micropropagation of lily cultivars, bulb scales were cultured on MS medium (4.3 g/L, Murashige and Skoog, 1962) supplemented with 9% sucrose, 0.1% myo-inositol, and 0.8% gelrite for 3 weeks according to a previous method (Jung et al., 1996) for proliferation of blub. After 3 weeks, to induce the leaves in the bulb, the bulb scales were subcultured on MS medium supplemented with 3% sucrose, 0.1% myo-inositol and 0.8% agar for 2 weeks. All the cultures were maintained at 24 ± 2 °C in a 16/8 h light (fluency density of 240 μmol $m^{-2}·s^{-1}$) / dark condition.

2.2. Preparation of fungal inoculum

The *B.elliptica* isolate (KACC43461) was obtained from the Korea Agricultural Culture Collection (KACC). The isolate was cultured on a 90 × 15 mm^2 petri dish (Hyundai Micro Co., Korea) containing 7.2% potato dextrose agar (PDA) (Becton, Dickinson and Co., USA) at 25 °C and 28 °C under a 12/12 h UV-A light/dark for 1, 2, and 3 weeks to induce sporulation. Then, 10 mL of sterile distilled water was added to the petri dish, which was raked with a sterilized spreader to suspend the mycelium. Fifteen microliters of the spore suspension in aliquot were stained with methylene blue and the concentration was measured using a hemocytometer on optical microscope (Zeiss, Germany). Spore suspensions were diluted with sterile distilled water to reach final concentrations of 2.0 × 10^2, 2.0 × 10^3, and 2.0 × 10^4 spores·mL^{-1}.

2.3. Detached leaf assay and disease assessment

Leaves of 2 weeks old *in vitro* grown lily cultivars were used for the detached leaf assay. The leaves were cut into 1.5 × 2.0 cm^2 pieces and washed twice with sterile distilled water to remove medium residue. One leaf piece was placed in a 85.40 × 127.60 × 20.20 mm^3 cell culture plate (SPL life sciences, Korea) containing a 3 mL aliquot of a spore suspension (2.0 × 10^2, 2.0 × 10^3, and 2.0 × 10^4 spores·mL^{-1}). Surfactants were not used to prevent concern about spore activity. The leaf discs were completely immersed in the spore suspension to be inoculated well. The cell culture plate was placed at 15 °C and 25 °C for 12 h in the dark in order to inoculate with *B. elliptica*. After incubation, leaves were washed with 70% ethanol and cultured on 0.6% agar plates at 15 °C and 25 °C in a 16/8 h light (fluency density of 130 μmol $m^{-2}·s^{-1}$)/dark. DI was observed at 3, 5, and 7 days after inoculation (DAI). The DI was measured by the extent of browning and rot in comparison to control leaves that were placed in sterile distilled water. The DI was divided into categories 0.00–4.00 according to leaf area infected: 0.00 = no lesions observed, 1.00 = 1–10%, 2.00 = 11–25%, 3.00 = 26–50%, 4.00 = over 50% leaf area infected (Hsieh and Huang, 1999). Then, the obtained DI was used to organize the lily cultivars into resistant or susceptible groups.

2.4. Statistical analysis

The detached leaf assay was repeated 3 times and 10 leaf pieces were inoculated in each experiment. The Student's *t*-test in Microsoft Office Excel 2013 (Microsoft Corporation, USA) was used to analyze the significance of each experiment.

2.5. Transcriptome resource and identification of putative candidate (R)-genes

Transcriptomic data from various *Lilium* inbred lines representing disease resistant (S20) and susceptible (S21) genotypes were obtained from the leaf transcriptome upon *Botrytis* infection, which is available in this laboratory (unpublished data). Raw data's are also available at National Agricultural Biotechnology Information Center (NABIC; http://nabic.rda.go.kr/) under accession numbers of NN-3727-000001 (S20) and NN-3728-000001 (S21). From these transcriptomic data,