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The pathogen-inducible promoter of defense-related *LsGRP1* gene from *Lilium* functioning in phylogenetically distinct species of plants

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ABSTRACT

A suitable promoter greatly enhances the efficiency of target gene expression of plant molecular breeding and farming; however, only very few promoters are available for economically important non-graminaceous ornamental monocots. In this study, an 868-bp upstream region of defense-related *LsGRP1* of *Lilium*, named P_{LsGRP1}, was cloned by genome walking and proven to exhibit promoter activity in *Nicotiana benthamiana* and *Lilium* 'Stargazer' as assayed by agroinfiltration-based β-glucuronidase (GUS) expression system. Many putative biotic stress-, abiotic stress- and physiological regulation-related *cis*-acting elements were found in P_{LsGRP1}. Serial deletion analysis of P_{LsGRP1} performed in *Nicotiana tabacum* var. Wisconsin 38 accompanied with types of treatments indicated that 868-bp P_{LsGRP1} was highly induced upon pathogen challenges and cold stress while the 131-bp 3′-end region of P_{LsGRP1} could be dramatically induced by many kinds of abiotic stresses, biotic stresses and phytohormone treatments. Besides, transient *GUS* expression in a fern, gymnosperms, monocots and dicots revealed good promotor activity of P_{LsGRP1} in many phylogenetically distinct plant species. Thus, pathogen-inducible P_{LsGRP1} and its 131-bp 3′-end region are presumed potential as tools for plant molecular breeding and farming.

1. Introduction

A promoter is a switch controlling transcription process of gene expression. Characterization of novel promoters not only expounds the mechanisms of gene transcriptional regulation but also enriches promoter resources for molecular breeding [1,2]. Because disease resistance of plants built up by overexpressing defense genes with a strong constitutive promoter probably results in low yields and poor quality, a pathogen-inducible promoter is therefore chosen to prevent physiological defects and energy wastage caused by unnecessary production of defense compounds [2,3]. By far, promoters commonly used in plant genetic engineering were mostly originated from dicots and graminaceous monocots; however, these promoters are less active in many non-graminaceous monocots, such as Liliaceae and Iridaceae [4–7] which include the genera of economically important ornamental bulbous plants. Besides, using a single promoter to drive multiple genes in the same individual often causes epigenetic silencing of target genes [8–11]. Thus, identification and characterization of promoters is requisite for molecular breeding, especially for non-graminaceous ornamental monocots.

Defense-related *LsGRP1* (*Lilium* 'Stargazer' glycine-rich protein 1) is a leaf-specific gene with increased expression pattern in *Lilium* exhibiting salicylic acid-induced systemic resistance against gray mold disease caused by the necrotrophic fungal pathogen *Botrytis elliptica* (Berkeley) Cooke [12–15]. The accumulation of LsGRP1 occurs in response to the pathogen challenge, presumably playing an important role in the induced resistance *via* its antimicrobial activity conferred by C-terminal region [16,17]. Besides, *LsGRP1* expression is consistent at all growth stages of lily, but enhanced in response to *B. elliptica* infection and the treatments of salicylic acid or probenazole [12,14,15,17]. The expression pattern of *LsGRP1* suggested that *LsGRP1* promoter has a basal expression level and can be activated in the presence of pathogens and resistance-inducing compounds [14,15,17].

Agroinfiltration is a versatile tool for transiently expressing foreign genes in plant tissues. After treated with phenolic compound acetosyringone, *Agrobacterium tumefaciens* is introduced into plant extracellular space through stomata using syringe or vacuum infiltration; then the recombinant T-DNA of binary vector harbored in *A. tumefaciens* is transferred into plant cells, and the gene(s) constructed in the T-DNA segment can be expressed transiently [18]. Since agroinfiltration is an efficient, rapid and reliable transient expression system of foreign genes, it becomes widely used in plant molecular biology research. Many plant promoters and transcriptional factors have been successfully characterized *via* agroinfiltration-based reporter gene expression systems. For

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examples, the enhancer-like positive regulatory sequence in rice *Osgrp-2* promoter [19], the arrangement of regulatory elements in *Arabidopsis NIA1* promoter [20], the virus- and salicylic acid-responsive *cis*-acting regulatory regions in tobacco *PR1a* and *myb1* promoters [21], and the activity of synthetic promoters [22]. In this study, *LsGRP1* promoter and its deletions were cloned and the expression profiles of *LsGRP1* promoter under the treatments of types of biotic and abiotic stresses or phytohormones, and in different plant species were investigated by agroinfiltration-based β-glucuronidase (GUS) expression system. Based on the results, the knowledge on the transcriptional regulation of *Lilium*, a non-graminaceous monocot, will be increased and the *LsGRP1* promoter regions can be a promising bioresource for plant molecular breeding and farming.

2. Materials and methods

2.1. Plant materials

Bulbs of Stargazer lily (*Lilium* oriental hybrid cv. Stargazer) with a perimeter of 14–16 cm were planted in a potting mix containing BVB Substrate No. 2 (Bas van Buuren, Maasland, Holland), vermiculite and perlite at a ratio of 6:1:1 (vol:vol:vol), and cultivated at 18 to 22 °C and 70 to 80% relative humidity with a light/dark cycle of 16 h/8 h for 8 weeks unless specifically indicated. Seedlings of Formosa lily (*Lilium formosanum*) were purchased from Yushi District Farmers' Association (Hualien, Taiwan), transplanted following the protocol for Stargazer lily and cultivated for three months. Seeds of *Nicotiana benthamiana*, *Nicotiana tabacum* var. Wisconsin 38, *Arabidopsis thaliana* ecotype Col-0, lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), Chinese kale (*Brassica oleracea* var. *alboglabra*) and common bean (*Phaseolus vulgaris*) were planted in the potting mix described above with a controlled-release fertilizer (Hi-Control 16-9-10, 180-type, Taiwan Horticultural Co., Ltd., Taipei, Taiwan), and cultivated at 23 to 28 °C with a light/dark cycle of 16 h/8 h for four to six weeks. *Zea mays* Honey Jean No. 3 (Know-You Co., Kaohsiung, Taiwan) was sown in a potting mix with BVB Substrate No. 2 and vermiculite at a ratio of 3:1 (vol:vol) and cultivated at 25 to 30 °C with a light/dark cycle of 12 h/12 h for four weeks. Other plant materials, including bird's nest fern (*Asplenium nidus*), Asian bayberry (*Nageia nagi*), ginkgo (*Ginkgo biloba*), Siam tulip (*Curcuma alismatifolia*), narcissus (*Narcissus hybrida*), tulip (*Tulipa gesneriana*), Asian taro (*Alocasia odora*), snake plant (*Sansevieria trifasciata*), butterfly orchid (*Phalaenopsis* sp.), dancing lady orchid (*Oncidium* sp.), petunia (*Petunia hybrida*), Madagascar periwinkle (*Catharanthus roseus*), African violet (*Sinningia speciosa*), coffee (*Coffea arabica*), basil (*Ocimum basilicum*), bottle gourd (*Lagenaria siceraria*), angled luffa (*Luffa acutangula*), chili pepper (*Capsicum annuum*) and bell pepper (*Capsicum annuum* var. *grossum*) were purchased from Taipei Pot Plant Auction (Taipei, Taiwan) and cultivated at 23 to 28 °C with a light/dark cycle of 16 h/8 h for one month.

2.2. Extraction of Lilium genomic DNA

Lilium genomic DNA was extracted using CTAB method [23] with modifications. Stargazer lily leaf tissue of 2 g was ground to a fine powder in liquid nitrogen and mixed with 8 ml 65 °C-CTAB extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 6% cetyltrimethyammonium bromide [CTAB], 1% polyvinylpyrrolidone, 20 mM ethylenediaminetetraacetic acid [EDTA], 300 μg/ml proteinase K, 3% 2-mercaptoethanoal, pH 8.0), incubated at 65 °C with a rotation at 6–8 rpm for 2 h, and gently extracted with equal volume of chloroform:octanol (24:1 [vol:vol]). The supernatant was collected by centrifugation at 6,000 × *g* for 10 min, treated with 1 mg/ml RNase at 37 °C for 20 min, mixed with 0.1-fold volume of CTAB/NaCl solution (10% CTAB, 0.7 M NaCl), incubated at 65 °C with a rotation at 6–8 rpm for 20 min, and then gently extracted with equal volume of chloroform:octanol. The steps of CTAB/NaCl solution and chloroform:octanol extractions were repeated for 3–5 times. The supernatant was extracted with equal volume of chloroform:octanol, then mixed with 0.7-fold volume of isopropanol and centrifuged at 6,000 × *g* for 10 min. The pellet of *Lilium* genomic DNA was washed with 80% ethanol, air dried and dissolved in sterile deionized water.

Table 1
Sequences of the primers used in this study.

Primer	Sequence
p275	5′-TGGTCGGCAACTCCTGCCTC-3′
p329	5′-CCTCAGCCAGCTCCCGACCAGCGTCGGAGG-3′
p367	5′-GGATGGGTACCTCATTTCTTCAAGC-3′
p370	5′-CACAGACAATAAGTGAATTAATAGCGTGGC-3′
p381	5′-CTGGATATCACGTTAAGGGGGCAATAGAGGATACTGAGG-3′
p383	5′-GTCTTCCGATTGATGCCACG-3′
OHP-*Kpn*I	5′-GAATTCGAGCTCGCCCGGGATCCTCTAGAGTAC-3′
OHP-*Pst*I	5′-GAATTCGAGCTCGCCCGGGATCCTCTAGATGCA-3′
OHP-*Sac*I	5′-GAATTCGAGCTCGCCCGGGATCCTCTAGAAGCT-3′
OHP-*Sac*II	5′-GAATTCGAGCTCGCCCGGGATCCTCTAGAGC-3′
AP	5′-GAATTCGAGCTCGCCCGGGAT-3′
NP	5′-GCTCGCCCGGGATCCTCTAGA-3′
pK11	5′-CGCTGCTACAACGGTTGC-3′
p170	5′-CTATGGGTGTCCATAAGCAGGCTC-3′
LsEF-1α-F	5′-AGTCTGGCGCATGTCCCTAA-3′
LsEF-1α-R	5′-GGAGCCTAAGTTTCTGAAGAATGG-3′

2.3. Cloning of LsGRP1 promoter

LsGRP1 promoter was obtained as follows. Firstly, the upstream of *LsGRP1* was amplified from genomic DNA of Stargazer lily using a genome walking kit by nested polymerase chain reaction (PCR). DW-ACP random primers of DNA Walking SpeedUp™ Premix kit (Seegene, Seoul, Korea), and the reverse primers specific to *LsGRP1* (p275 and p329) and its upstream region (p367 and p370) were used. The amplicons were cloned into pGEM-T Easy vector (Invitrogen, Carlsbad, California, USA) and sequenced. Then a primer-based approach for genome walking [24] was followed to clone the further upstream region. The genomic DNA of Stargazer lily was digested with restriction enzyme *Kpn*I, *Pst*I, *Sac*I and *Sac*II (Roche Inc., Basel, Switzerland), and ligated with overhanging adapter-primer OHP-*Kpn*I, OHP-*Pst*I, OHP-*Sac*I and OHP-*Sac*II, respectively. The upstream region of *LsGRP1* was amplified from the ligation products by nested PCR using OHP-specific primers (AP and NP) and the reverse primers specific to the upstream region of *LsGRP1* (p381 and p383). The amplicons were cloned into pGEM-T Easy vector and sequenced. The primer sequences are listed in Table 1.

2.4. Computational analysis

Putative TATA box and transcription start site were predicted using plant promoter identification program TSSP [25,26]. Putative plant *cis*-acting regulatory DNA elements were predicted using database PLACE [27].

2.5. Construction of recombinant vectors with LsGRP1 promoter regions

Binary vector pBI121 (Clontech Laboratories, Inc., California, USA) was digested with *Hin*dIII and *Bam*HI (Roche Inc.), and the linearized pBI121 fragment without the sequence of *Cauliflower mosaic virus* 35S promoter [28] was recovered and treated with Klenow enzyme (New England Biolab, Massachusetts, USA) to