Plant Science 215–216 (2014) 124–133

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Identification of the tapetum/microspore-specific promoter of the pathogenesis-related 10 gene and its regulation in the anther of *Lilium longiflorum*☆

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ARTICLE INFO

Article history:
Received 10 July 2013
Received in revised form
20 September 2013
Accepted 7 November 2013
Available online 15 November 2013

Keywords:
Hormone crosstalk
Microspore
Pathogenesis-related 10 (*PR10*)
Tapetum-specific promoter

ABSTRACT

A tapetum/microspore-specific *pathogenesis-related* (*PR*) *10* gene was previously identified in lily (*Lilium longiflorum* Thunb.) anthers. *In situ* hybridization and RNA blot analysis indicated that the lily *PR10* genes are expressed specifically and differentially in the tapetum of the anther wall and in microspores during anther development. The accumulation of *PR10* transcripts was exogenously induced by gibberellic acid (GA) and was suppressed by ethylene. Studies using inhibitors of GA and ethylene revealed that the lily *PR10* is modulated by an antagonistic interaction between GA and ethylene. The treatment of norbornadien, an ethylene inhibitor, caused the tapetum to become densely cytoplasmic and highly polarized, whereas uniconazole, an inhibitor of GA biosynthesis, arrested tapetal development to a status close to that of control. The expression of the lily *PR10g* promoter in transgenic Arabidopsis was determined using the *β-glucuronidase* (*GUS*) reporter gene indicated that the decisive fragment required for anther specificity is located −1183 bp to −880 bp upstream of the transcription start site. The *PR10gPro::barnase* transgenic lines exhibited complete male sterility because of the disruption of the tapetum and the deformation of microspore/pollen. The anther specificity of lily *PR10* highlights the importance of the tapetum/microspore-specific *PR10g* promoter for future biotechnological and agricultural applications.

1. Introduction

Plants grown in natural environments are often attacked by a variety of pathogens. In response to infections caused by pathogens, plants express various proteins such as pathogenesis-related (PR) proteins. Plant PR10s are small (15–18 kDa), acidic, and intracellular proteins [1] distributed in a variety of broad-spectrum species from gymnosperms [2] to dicots [3,4] and monocots [5,6]. Seventeen families of PR proteins have been identified based on structural and their functional properties [7]. PR proteins exhibit a variety of functions; however, the functions of PR1 and PR17 are still unknown. PR10s are reportedly important for plant disease resistance and they exhibit ribonuclease-like activity against viral RNA [8]. In addition to this pathogen-defense, some PR10 proteins exhibit other functions. A pea *PR10* gene that encodes ABR17 enhances tolerance against multiple abiotic stresses [9]. Crystal structure analysis of Bet v 1 in birch revealed that it contains a hydrophobic cavity that may participate in the transfer of apolar ligands. Alternatively, Bet v 1 may interact with brassinosteroids (BRs), which is involved in plant growth and development [10]. PR10 proteins are developmentally regulated in different plant tissues and organs [1], including anthers [5], pollen grain [11], seeds [9], and vegetative organs [12]. OsPR10 from rice may be crucial for embryonic development and seed germination [13]. The PR10 protein from *Brassica napus* enhances seed germination and seedling growth under saline conditions [14].

PR10 genes are regulated by various hormones. *OsPR10* from rice is induced by gibberellic acid (GA) and is suppressed by abscisic acid (ABA) during seed germination [13]. The rice *PR10* gene that encodes PBZ1 is negatively regulated by ABA through the mediation of a mitogen-activated protein kinase [15]. Wang et al. [16] previously reported that aside from inducing the expression of the lily *PR10* gene by activating JA, ABA participates in a JA-independent pathway involving one or more staurosporine-sensitive protein kinases in the anther. The gene expression regulated by an intricate interaction of various hormones has begun to unravel, but the hormonal regulation network remains unclear.

PR10 promoters have been cloned and functionally analyzed in parsley [17], bean [18], potato [19], and western white pine [12]. The bean *Ypr10c* promoter drives *β-glucuronidase* (*GUS*)

☆ The promoter sequence of lily *PR10g* has been deposited in GenBank under accession no. KC815691.

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0168-9452/$ – see front matter © 2013 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.plantsci.2013.11.006

expression in an organ-specific, dark-dependent, SA-inducible manner [18]. A positive *cis*-regulatory region of the potato *PR10a* gene that interacts with phosphorylated PR10a binding factor 1 was identified to be responsible for fungal or wound induction [19]. The two regions of the western white pine *PmPR10-1.13* promoter are responsible for disease and wound inducible expression [12]. Several *cis*-regulatory elements of the *PmPR10-1.13* gene have been identified, including the elicitor responsive element (EIRE)-like sequence [20], JA-responsive motifs [21], and ethylene-responsive enhancer element (ERE)-like motif [22]. However, little information has been reported regarding the *cis*-regulatory elements of the *PR10* gene responsible for floral organ-specific expression.

Lily *PR10* are anther-specific genes that include two distinctive subgroups [5,16]. We have further demonstrated that *PR10* is expressed in the tapetum and microspore. To elucidate the feature of anther specificity, we examine the mode of expression regulated by hormones and analyze the function of a member of *PR10* gene promoters, *PR10g*, in Arabidopsis. *PR10* expression is modulated by the antagonistic crosstalk between GA and ethylene. The tapetum/microspore specificity of the lily *PR10g* gene is determined both in lily anthers and in transgenic Arabidopsis.

2. Materials and methods

2.1. Plant materials and treatments

Lily (*Lilium longiflorum* Thunb. cv. Snow Queen) plants were grown in the field. Buds ranging from 20 to 75 mm were dissected to isolate anthers. Meiosis occurred in the pollen mother cells at bud size around 20–25 mm, resulting in the formation of tetrads. Afterwards, microspore mitosis was complete at bud size around 65–70 mm and pollen subsequently entered the maturation phase of development. Concomitant with the development of microspore, the tapetum of the anther wall became secretory and then degenerated afterwards. The anther was separated from the filament in buds longer than 25 mm; otherwise, the two organs were combined. All materials were stored at −80 °C until use. GA_3, ethephon (Sigma–Aldrich, St. Louis, MO, USA), uniconazole (Wako Pure Chemical Industries Ltd., Osaka, Japan), and norbornadien (NBD) (Sigma–Aldrich, St. Louis, MO, USA) were used in indicated experiments.

2.2. RNA in situ hybridization

Anthers of indicated lily bud sizes were fixed at 4 °C for 16 h under vacuum in 1× PBS buffer (130 mM NaCl, 7 mM Na_2HPO_4 and 3 mM NaH_2PO_4, pH 7.4) containing 4% paraformaldehyde, 0.25% glutaraldehyde, and dehydrated and then embedded in Steedman's wax. The embedded tissues were sectioned (7–10 μm) in a microtone and affixed to poly-L-lysine-coated slides. Digoxigenin (DIG)-labeled RNA probes were synthesized using a DIG RNA labeling kit (SP6/T7) (Roche Diagnostics GmbH, Penzberg, Germany). *In situ* hybridization was carried out essentially according to the DNA *in situ* hybridization protocol as described [23] with some modifications. Given that *PR10g* is closely related to other members of *PR10* [16], the positive signal detected indicates a group of *PR10* transcripts, but not *PR10g* only. Hybridization sites were immunologically detected by using an alkaline phosphatase-conjugated anti-DIG antibody diluted in 1:500 with 1× TNB buffer [0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl and 0.5% blocking reagent (Roche Diagnostics GmbH, Penzberg, Germany)]. Colorization was left to develop for 2 h with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium as substrates of alkaline phosphatase. The hybridization signal viewed under a bright-field microscope is brownish purple. Sections were counterstained with 0.001% Fast Green.

2.3. RNA blot analysis

To separate microspores from the anther wall, anthers of young buds were sliced open transversely with a scalpel. Microspores were gently squeezed out into a buffer of 10 mM sodium acetate, pH 5.2. After centrifugation at 5000 × *g* for 3 min, the pellet (microspores) was ready for the extraction of total RNA. Total RNA extracted from developing anthers was electrophoresed in 1.0% formaldehyde/3-[N-morpholino] propanesulfonic acid agarose gels and transferred onto nylon membranes (Immobilon-Ny^+ membrane; Millipore, Bedford, MA, USA). The membranes with immobilized RNA were prehybridized for 4 h at 42 °C in medium containing 5× SSC (0.75 M NaCl and 75 mM sodium citrate, pH 7.0), 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 20 mM sodium phosphate, pH 6.5, 0.1% (w/v) SDS, 1% glycine, 50% formamide and 150 μg mL^{-1} of denatured salmon sperm DNA using standard procedures [24]. For hybridization, the prehybridization solution was removed and replaced with hybridization buffer that contained the same components as the prehybridization buffer but lacked 1% glycine. Denatured salmon sperm DNA (100 μg mL^{-1}) and random-primed ^{32}P-labeled probe (1.0×10^9 cpm μg^{-1}) were added to the hybridization buffer. Hybridization was carried out at 42 °C overnight with constant agitation. The membranes were washed twice at 50 °C in 2× SSC, 0.1% (w/v) SDS for 20 min, followed by twice at 60 °C in 0.1× SSC, 0.1% (w/v) SDS for 20 min. The membrane was visualized using phosphoimager plates using either one or two intensifying screens (Sigma–Aldrich, Saint Louis, MO, USA) for 3 d or less.

2.4. Quantitative real-time PCR (qRT-PCR) analysis

For qRT-PCR, the cDNA was amplified with *rRNA*- and *PR10g*-specific primers (Supplementary Table S1) in the presence of SYBR Green I Nucleic Acid Gel Stain (Lonza, Rockland, ME, USA) 10,000× dilution from stock using a Rotor-Gene 3000 (QIAGEN (Corbett), Manchester, UK). Amplification of *rRNA* under identical conditions was used as an internal control to normalize the level of cDNA. The data obtained were analyzed with Rotor-Gene 6 software (Corbett). Since SYBR Green I dye binds to the minor groove of any double stranded DNA, including specific products, nonspecific products, and primer dimers, it is necessary to perform a melting curve analysis at the end of each qRT-PCR experiment. Nonspecific products or primer dimers can be identified as they melt at a lower temperature compared to the specific amplicon. Specific temperatures obtained for *rRNA* (64 °C) and *PR10g* (64 °C) validated the specific product formation. qRT-PCR experiments were repeated three times independently and samples in each experiment are made in duplicate.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.11.006.

2.5. Isolation of PR10g promoter

For the isolation of the promoter region of lily *PR10g* [16], thermal asymmetric interlaced (TAIL)-PCR strategy was employed [25]. A set of nested sequence specific primers, PR10-R2, PR10-R3 and PR10-R4 (Supplementary Table S1), was utilized in successive reactions together with a random hexamer for the amplification of the promoter of *PR10g*. Primary TAIL-PCR reactions (50 μL) contained 1× PCR buffer (10 mM Tris–HCl, pH 9.5, 50 mM KCl, 0.1% Triton X-100), 25 mM $MgCl_2$, 2 mM of each dNTP, 200 ng of lily genomic DNA, 20 μM PR10-R2, 20 μM random hexamer, and 2.5 U of Taq polymerase (MDBio, Taipei, Taiwan). Primary TAIL-PCR was