



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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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.

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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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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. GA₃, 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 $1\times$ PBS buffer (130 mM NaCl, 7 mM Na₂HPO₄ and 3 mM NaH₂PO₄, 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 microtome 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 $1\times$ 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 $5000\times g$ 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 $5\times$ 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 $150\mu\text{g mL}^{-1}$ 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 ($100\mu\text{g mL}^{-1}$) and random-primed ³²P-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 $2\times$ SSC, 0.1% (w/v) SDS for 20 min, followed by twice at 60°C in $0.1\times$ SSC, 0.1% (w/v) SDS for 20 min. The membrane was visualized using phosphorimager 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) $10,000\times$ 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 $1\times$ PCR buffer (10 mM Tris–HCl, pH 9.5, 50 mM KCl, 0.1% Triton X-100), 25 mM MgCl₂, 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

executed as follows: 94 °C for 10 min, 32 cycles of 94 °C for 1 min, annealing at 69 °C for 30 s and elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min, using a GeneAmp PCR System 2400 (Perkin-Elmer, Waltham, MA, USA). Aliquots (5 µL) of the primary PCR products were directly subjected to secondary TAIL-PCR reactions (50 µL) containing 1× PCR buffer, 25 mM MgCl₂, 2 mM of each dNTP, 200 ng of lily genomic DNA, 20 µM PR10-R3, 20 µM random hexamer, and 2.5 U of Taq polymerase. After amplification, aliquots (5 µL) of the secondary PCR products were re-amplified in tertiary TAIL-PCR reactions (50 µL). Components and their concentrations were the same as in the secondary reaction except that PR10-R4 replaced RP10-R3. Amplified products from the reactions were fractionated and either stained with ethidium bromide or blotted onto nitrocellulose membranes. The membranes were prehybridized and then hybridized with an internal probe corresponding to the 5'-end of known *PR10g* sequence, which had been ³²P-labeled (1.0×10^9 cpm µg⁻¹) by random priming using the Rediprime™ II Random Prime Labeling System (Amersham Biosciences). Prehybridization and hybridization were carried out as described above for RNA blot analysis. The DNA fragments on the gel corresponding to the positive hybridization signals were eluted and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The recombinant plasmid in which the *PR10g* promoter resided was isolated using a Plasmid Miniprep Purification kit (GeneMark Technol. Co. Ltd, Taipei, Taiwan) and sequenced with an ABI 3730XL DNA analyzer (Foster City, CA, USA) by Mission Biotech Co. Ltd. (Taipei, Taiwan). Analysis of the promoter region was carried out using the PLACE database available at <http://www.dna.affrc.go.jp/htdocs/PLACE/>.

2.6. Constructs of *PR10gPro::GUS* and *PR10gPro::barnase* and plant transformation

Both *PR10gPro::GUS* and *PR10gPro::barnase* were constructed into pBI101 vector. For *PR10gPro::GUS* construction, the *PR10g* promoter with a *Hind*III site at the 5' end and a *Bam*HI site at the 3' end was amplified by PCR using a pair of primers: Z7 T3g-*Hind*III and Z7 T7g-*Bam*HI (Supplementary Table S1) and *PR10g* promoter as a template. The amplified *PR10g* promoter fragment was digested with *Hind* III and *Bam* HI and ligated with pBI101 which was digested with the same restriction enzymes to generate *PR10gPro::GUS* construct. Similar strategy was used for *PR10gPro* deletion mutants::*GUS*, and the 5' primer was instead of Z7 T3g-*Hind*III-1, Z7 T3g-*Hind*III-2 or Z7 T3g-*Hind*III-3 (Supplementary Table S1). For *PR10gPro::barnase* construction, the *barnase* fragment with a *Bam*HI site at the 5' end and a *Sac*I site at the 3' end was used to replace the *GUS* of *PR10gPro::GUS* construct and amplified by PCR using a pair of primers: bn T3-*Bam*HI and bn T7-*Sac*I (Supplementary Table S1) and *barnase* vector as a template. The amplified *barnase* fragment digested with *Bam*HI and *Sac*I was ligated with *PR10gPro::GUS* construct which was digested with the same restriction enzymes to generate *PR10gPro::barnase* construct. The primers for *barnase* were designed based on the published sequence of *barnase* gene [26]. All constructions were sequenced, transformed into *Agrobacterium tumefaciens* strain GV3101 and then transformed into *Arabidopsis* by using the floral dip method [27].

2.7. *GUS* assay and histological analysis

GUS activity was analyzed by histochemical staining using the substrate 5-bromo-5-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) as described [28] with some modifications. Seedlings, whole plants or buds were fixed in 90% acetone for 20 min and immersed in a X-gluc solution in 50 mM sodium phosphate, pH 7.2, 0.5 mM each potassium ferricyanide and potassium ferrocyanide, and 2 mM X-gluc. The reactions were incubated at 37 °C for 12 h.

For histological analysis, samples were fixed in FAA (45% absolute ethanol, 5% glacial acetic acid, 5% formaldehyde) solution and vacuum was applied for 30 min at room temperature. Dehydration was performed with t-butanol and ethanol and then embedded in paraffin. Sections were obtained with a microtome and stained with 0.1% toluidine blue.

3. Results

3.1. *In situ* localization of *PR10* mRNA in developing anthers of *L. longiflorum*

RNA *in situ* hybridization with DIG-labeled antisense RNA probes of the lily *PR10g* gene was performed to determine the cellular location of *PR10* transcripts. No positive signal was observed in sections of anthers from 20 to 50-mm buds which were treated with sense probes (Fig. 1). On the contrary, when the DIG-labeled antisense riboprobe of *PR10g* was used, strong dark brown hybridization signals were observed in the tapetum of both 30- and 40-mm buds (Fig. 1). At this time, the tapetal cells became polarized and highly secretory in the anther. Given that *PR10g* was closely related to other members of *PR10* [16], the positive signal detected in the tapetum indicated that a group of *PR10* transcripts significantly expressed in the tapetum. However, hybridization signals in the microspores were not discernible due to the structure distortion. As buds grew to 50 mm, the tapetal cells began to disintegrate in the anther and the hybridization signal strength decreased compared to that observed in the 30- and 40-mm buds, suggesting significant down-expression of lily *PR10* gene expression (Fig. 1). It is consistent with an earlier report wherein the lily *PR10* genes were only expressed in the phase of microspore development [5].

3.2. Temporal accumulation of *PR10* transcripts in developing microspore of *L. longiflorum*

To further determine whether the lily *PR10* genes are expressed in microspore or not, anthers of various size classes were dissected into two parts: the anther wall and the microspores. Anthers of young buds were sliced open transversely with a scalpel and microspores were gently squeezed out for total RNA extraction. The blots of total RNA isolated from anther, anther wall and microspore of 30-mm buds and from the microspores/pollen of various anther developmental stages were hybridized with ³²P-labeled *PR10g* cDNA. In addition to detection in the anther wall (tapetum), *PR10* transcripts were coordinately detected in the various stages of microspores in buds up to 45 mm (Fig. 2). No signal was detected from blots of mRNA isolated from the microspore/pollen within 65 mm buds and thereafter, further indicating the stage-specificity of lily *PR10* gene expression in the anther. Signals detected in the anther and anther wall were used as control.

3.3. Effects of GA and ethephon on the accumulation of *PR10* transcripts in young anthers of *L. longiflorum*

Plant hormones were reported to be involved in the regulation of early anther development [29,30]. To investigate the inductive effect of GA on expression of the lily *PR10* genes, the application of exogenous GA was used. Lily plants with 18–22 mm-long buds were dipped in solutions containing various concentrations of GA₃ for 24 h. qRT-PCR analysis revealed that *PR10g* gene increased its level of expression as 100 µM GA₃ was applied (Fig. 3A). Little inductive accumulation of *PR10g* transcripts was detected when 1 or 10 µM GA₃ was applied. In order to determine the kinetics of the GA₃-stimulated accumulation of *PR10* transcripts in lily anthers, a time-course experiment was conducted. Lily plants with 18–22-mm buds were dipped in a solution without or with 100 µM GA₃

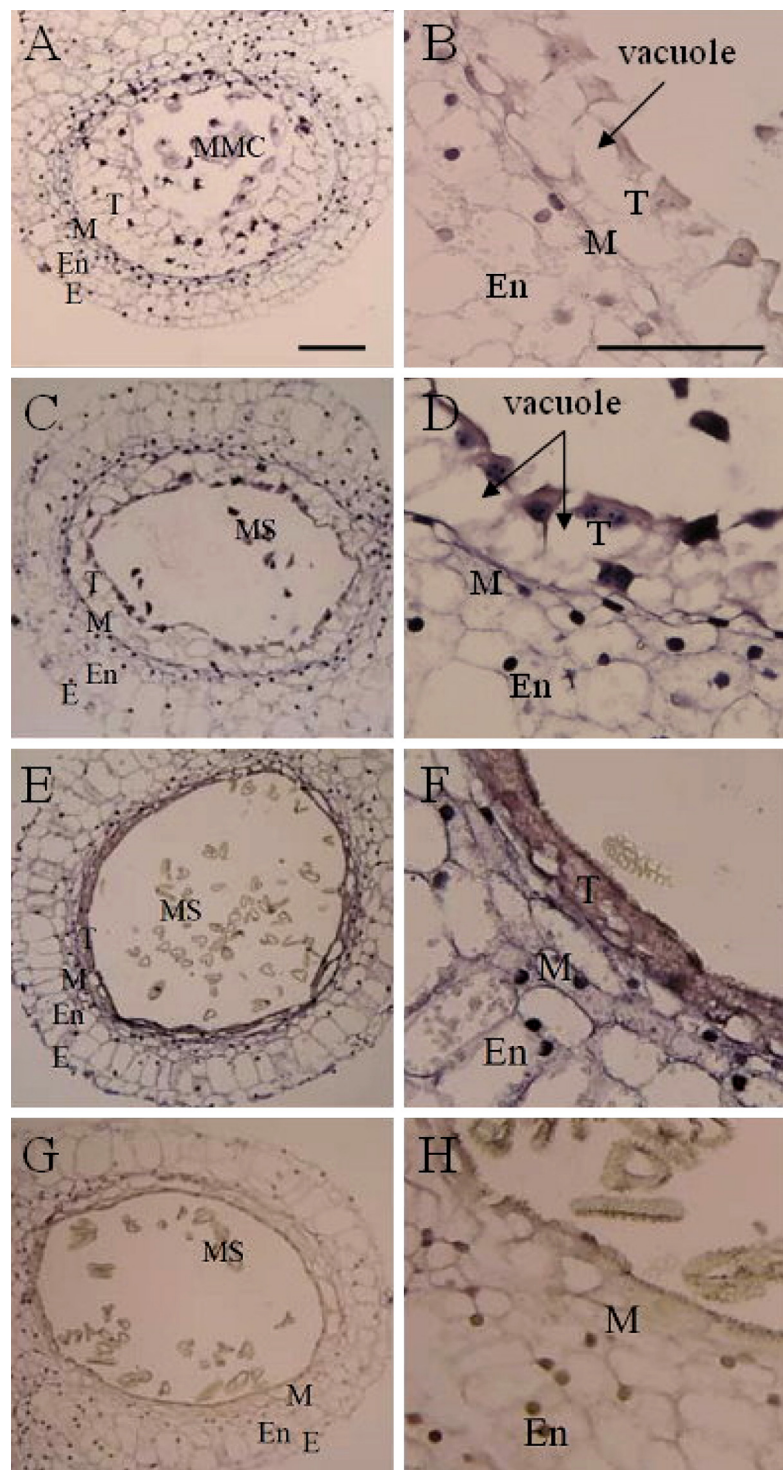


Fig. 1. *In situ* hybridization of *PR10* transcripts in the developing anthers of *Lilium longiflorum*. The expression of *PR10* was analyzed in bright-field illuminated 7–10-mm cross-sections of lily anthers of 20 mm (A), 30 mm (B–D), 40 mm (E and F), and 50 mm (G and H) buds using DIG-labeled sense (B) and antisense (A and C–H) *PR10g* RNA probes. E, epidermis; En, endothecium; M, middle layer; MMC, microspore mother cell; MS, microspore; T, tapetum. Bar = 100 μ m.

for various time intervals. qRT-PCR analysis showed that the inductive accumulation of *PR10g* transcripts was observed after 24 h of GA_3 treatment (Fig. 3B). Similar observations were also obtained from RNA blot analysis (Fig. 3). These results indicated that the expression of lily *PR10* genes were modulated by exogenous GA_3 .

To investigate the effect of ethylene on *PR10* gene expression, the application of exogenous ethephon, an ethylene-generating compound, was used. Lily plants with 18–22-mm buds were dipped in solutions containing various concentrations of ethephon for

24 h. qRT-PCR analysis showed that a decreasing level of *PR10g* transcripts was observed when various exogenous ethephon were applied (Fig. 3A). In order to determine the kinetics of transcript accumulation of *PR10* affected by ethephon in lily anthers, a time-course experiment was conducted. Lily plants with 18–22-mm buds were dipped in a solution without or with 100 μ M ethephon for various time intervals. qRT-PCR analysis showed that the *PR10g* transcript decreased its level of accumulation after 24 h of ethephon treatment (Fig. 3B). Similar observations were also obtained

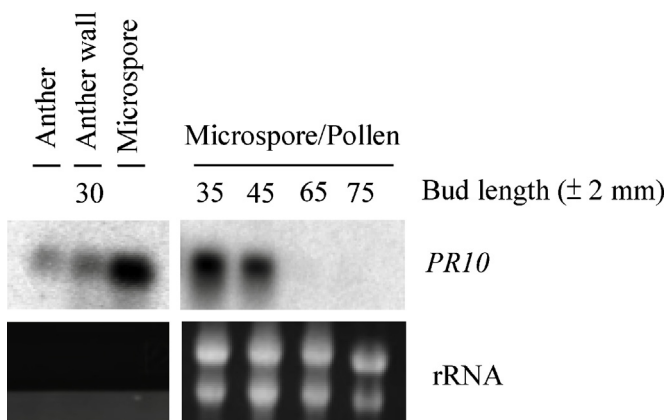


Fig. 2. Differential expression of *PR10* in developing microspores of *Lilium longiflorum*. Total RNA (20 μ g) were isolated from the anther, anther wall and microspores of 30-mm buds and from the microspore/pollen of 35-, 45-, 65-, and 75-mm buds during the phase of microspore development. Total RNA was denatured, fractionated on formaldehyde-agarose gels, transferred to nylon membranes and hybridized to the ^{32} P-labeled *PR10g* cDNA. Almost equal amounts of total RNA were loaded in each lane, as determined by ethidium bromide staining of the gel.

from RNA blot analysis (Fig. 3). These results indicated that the lily *PR10* gene was down-modulated by exogenous ethephon.

3.4. The antagonistic crosstalk between GA and ethylene regulates the expression of *PR10* in young anthers of *L. longiflorum*

GAs are produced in the tapetum [31]. To further examine whether *PR10* is stimulated by endogenous GA and other hormones in anthers, the treatments with uniconazole, a potent inhibitor of GA biosynthesis [32] and NBD an effective inhibitor of ethylene action [33] were applied. Because the *PR10g* mRNA was first detected around 20-mm buds and accumulated to maximum levels at the later stages of microspore development [34], we chose anthers of buds around 20 mm for inhibition analysis. Lily plants with 18–22 mm buds were dipped in a solution with or without 100 μ M uniconazole and/or NBD and continuously grown to 23–26 mm (about 4 d) after which total RNA was extracted from the anther. qRT-PCR and RNA blot analyses revealed that without the addition of inhibitors, the *PR10g* mRNAs accumulated in the anther after buds grew from 18–22 to 23–26 mm (Fig. 4A). When 100 μ M uniconazole was added, mRNA accumulation was inhibited at a modest level, reflecting that the *PR10g* gene was induced by endogenous GA. The 4 d treatment of uniconazole arrested tapetal development to a status similar to that of control (Fig. 4B). When 100 μ M NBD was applied, the accumulation of *PR10g* mRNAs significantly increased, suggesting that expression of *PR10g* is negatively modulated by ethylene (Fig. 4A). Similar observations were also obtained from RNA blot analysis (Fig. 4A). This strongly suggests that a crosstalk between GA and ethylene is involved in the regulation of *PR10* genes occurring in young anthers. The treatment of NBD did not cause any visible growth retardation of lily buds. On the contrary, the tapetum in the anther became densely cytoplasmic and highly polarized such that the vacuole was toward the outside and the cytoplasm toward the locular side (Fig. 4B). It is obvious that the tapetal cells grew advanced than that of control. When plants were treated with uniconazole supplemented with NBD, *PR10* mRNA significantly decreased its level of accumulation comparing to the one treated with NBD only (Fig. 4A) but the tapetal morphology was similar to the one treated with uniconazole only (Fig. 4B). It reinforces the concept that GA is the main hormone modulating *PR10* expression and ethylene negatively modulates GA-induced expression of the lily *PR10* genes.

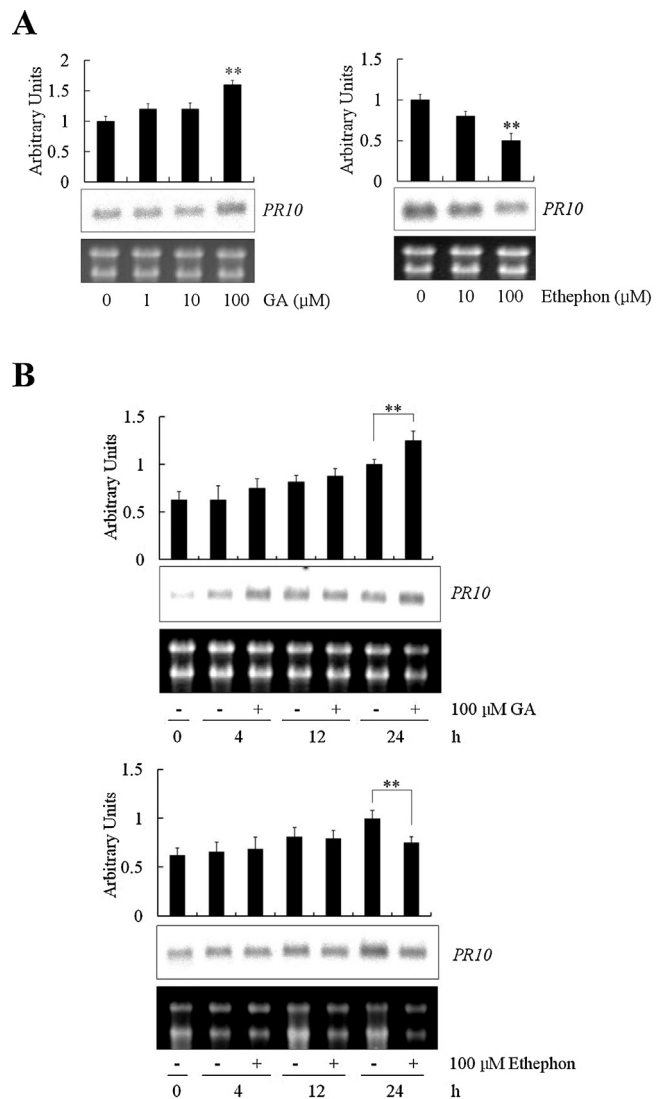


Fig. 3. The expression of lily *PR10* genes stimulated by GA₃ or ethephon. Freshly cut lily plants with bud sizes of 18–22 mm were dipped in solution without or with the addition of (A) various concentrations of GA₃ or ethephon for 24 h, respectively or (B) 100 μ M GA₃ or ethephon for 0, 4, 12, and 24 h. Total RNA was isolated from the anther. The levels of *PR10g* and *PR10* mRNAs were determined by qRT-PCR (upper panel) and RNA blot hybridization (lower panel), respectively. The data were obtained from three independent experiments. Error bars represent SD (*t*-test: ** $P < 0.01$). For RNA blot analysis, RNA samples (20 μ g) were denatured, fractionated on formaldehyde-agarose gels, transferred onto membranes and hybridized to the ^{32} P-labeled *PR10g* cDNA. Almost equal amounts of total RNA were loaded in each lane, as determined by ethidium bromide staining of the gel (bottom).

3.5. The lily *PR10* proteins do not have a typical P-loop motif

The identified lily *PR10* proteins have been categorized into two subclasses [16]. As shown in Fig. 5A, the lily *PR10g* and *d* are representatives of the two subclasses. The alignment of *PR10* proteins of various species revealed a conserved P-loop motif in the sequences (Fig. 5B). The P-loop motif presumably functions as a binding site for nucleotides and confers ribonuclease activity to the protein [1]. However, all lily *PR10* proteins including the two representatives do not have a typical P-loop motif.

3.6. *GUS* expression driven by the lily *PR10g* promoter in transgenic *Arabidopsis*

The TAIL-PCR strategy was employed to obtain the 5'-regulatory region of the lily *PR10g* gene (Fig. 6). The *PR10g* promoter

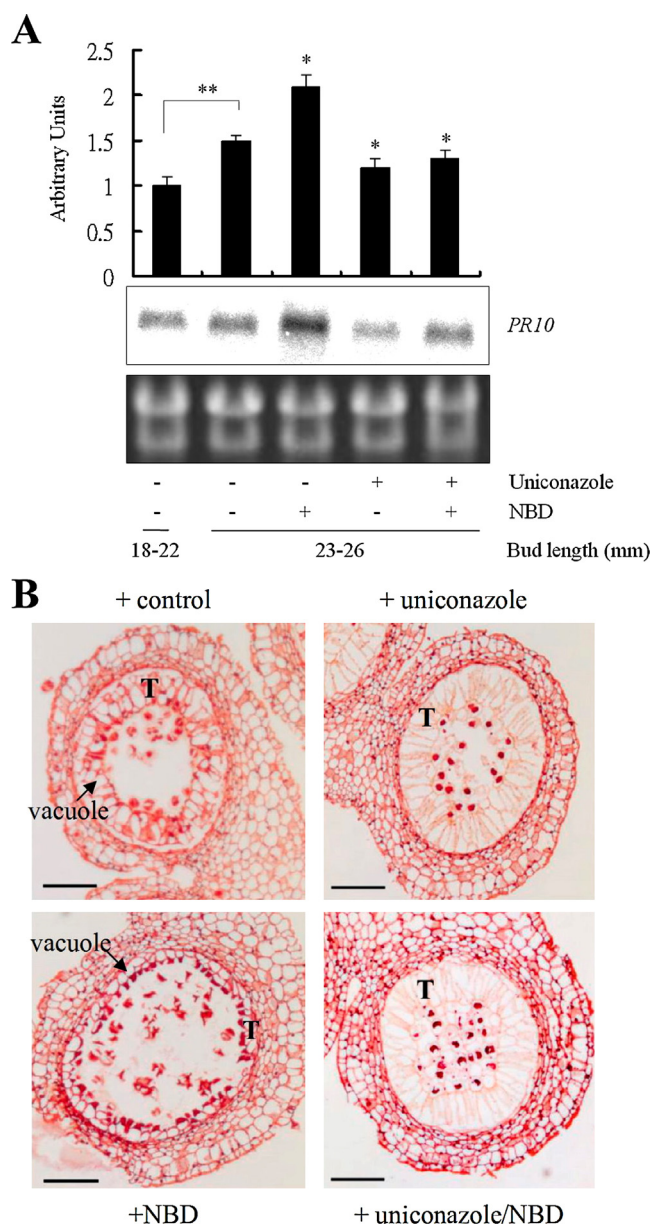


Fig. 4. Effects of GA and ethylene inhibitors on the expression of *PR10* genes in developing anthers of *Lilium longiflorum*. (A) Freshly cut lily plants with bud sizes of 18–22 mm were dipped in a solution without or with the treatment of 100 μ M uniconazole, an inhibitor of GA biosynthesis, and/or NBD, an inhibitor of ethylene activity for 4 d at which time the bud size continuously grew to 23–26-mm in length. Buds were decapitated and anthers within buds were dissected. Total RNA was isolated from the anther. The levels of *PR10g* and *PR10* mRNAs were determined by qRT-PCR (upper panel) and RNA blot hybridization (lower panel), respectively. The data were obtained from three independent experiments. Error bars represent SD (t -test: * $P < 0.05$, ** $P < 0.01$). For RNA blot analysis, RNA samples (20 μ g) were denatured, fractionated on formaldehyde-agarose gels, transferred onto membranes and hybridized to the ^{32} P-labeled *PR10g* cDNA. Almost equal amounts of total RNA were loaded in each lane, as determined by ethidium bromide staining of the gel (bottom). (B) Transverse sections of anthers of 20-mm buds treated without or with 100 μ M uniconazole and/or 100 μ M NBD for 4 d. Sections were stained with 1% safranin O in 50% ethanol. T, tapetum. Bar = 200 μ m.

(accession no. KC815691) extending from the lily *PR10g* (accession no. AF021853) contains a 50bp fragment of the 5'-untranslated region. The presumed TATA box sequence is present at 31bp upstream of the putative transcriptional start site. Sequence analysis of the promoter region showed the presence of three possible pollen- and/or anther-specific *cis*-regulatory motifs (Fig. 6), including a GAATTTGTTA-like motif, TAAAATGTTA, at position nucleotide

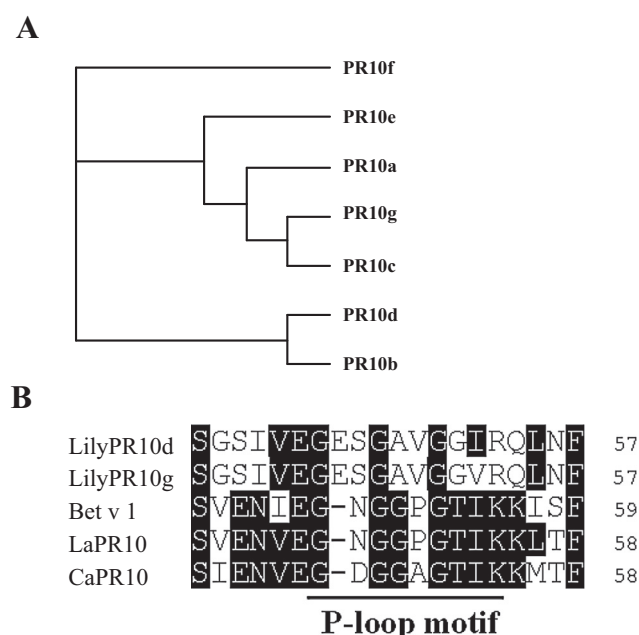


Fig. 5. The P-loop motif in PR10 proteins. (A) Phylogenetic analysis of the lily PR10 proteins. PR10a (accession no. AAD17335), PR10b (accession no. AAD17335), PR10c (accession no. AAF21622), PR10d (accession no. AAF21623), PR10e (accession no. AAF21624), PR10f (accession no. AAF21625), and PR10g (accession no. AAD17336). (B) The P-loop motifs of lily PR10d and PR10g amino acid sequences are compared with the predicted amino acid sequences of related PR10 proteins: birch Bet v1 (accession no. CAA96538), lupine LaPR10 (accession no. CAA03926), and hot pepper CaPR10 (accession no. AAF63519). A dash in the sequence indicates a gap introduced in order to maintain good alignment. The conserved P-loop motif (GxGGxGxxK) is underlined. That more than two of those amino acid residues are identical among sequences is highlighted in black blocks. The Multiple Sequence Alignment application (AlignX) of the Vector NTI Suite program (InforMax, Inc., North Bethesda, MD) based on the Clustal W algorithm was used.

–211 to nucleotide –202, and GTGA and AGAAA [35,36] which were found to be present in multiple copies in the 5' region of *PR10g*. Besides, the promoter region also contains various potential motifs related to hormone and stress responses (Table 1).

The lily *PR10g* promoter was fused with the *GUS* reporter gene (*PR10gPro::GUS*) and introduced into wild-type *Arabidopsis* plants to examine the expression pattern and function of *PR10g*. Of the 10 *PR10gPro::GUS* transgenic lines, eight had similar patterns of *GUS* stain during anther development. We just showed the data of one representative line. *PR10g* was strongly and specifically expressed in young anthers (Fig. 7A), but not in the anther of mature flowers and in seedlings and vegetative organs of the *PR10gPro::GUS* transgenic lines (Supplementary Fig. S1).

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>.

The 1183bp promoter fragment was subjected to a series of 5'-deletion analyses to further determine the functional characterization of the lily *PR10g* promoter. Three progressively deleted fragments were constructed (Fig. 7B), and fused upstream of the *GUS* reporter gene. The constructs were then introduced into *Arabidopsis* for promoter functional assays. *GUS* activity was detected in the young anthers of transgenic lines when the *GUS* gene was driven by the construct of the *PR10g* promoter with full-length (Figs. 7A and B). However, no signal was detected in the anther when the fragment was deleted down to –879bp upstream of the putative transcription start site. Similarly, no detectable signal was observed with the other two deletion constructs, –546 and –197bp upstream of the putative transcription start site (Fig. 7B). The lack of signal indicates that the promoter fragment

-1183 gat gctacaggag atcactcgat gtatatgtgt
 -1150 tgatttagtac aatcagggcc acatagagggt atcccttcag gataacaaca
 -1100 ttcttaactg atttgttctc tcataaatca acattcaatc ctattttgga
 -1050 gttacatata taaccttttg acatcaaaat attattatca taagttctag
 -1000 aagatatata taagtccttag tgccatttca tccatgttcc tcgtggttat
 -950 cttataagag cgaaccatgg tcgatggtga tgtggaggac aaggtatatt
 -900 gggtccttgg aatttaggtc tctttcgagc tttagcgggt tatatttgtt
 -850 ttatatattct gtaatcgac ttgttcacat gcatcatatt aataaaatta
 -800 taatcttatt ttctctcttg ttgtgtcgtg ccaatttaaa atattttttt
 -750 gtttgcattg aagattttat gtgtgtgtag atgagtaatg acactttatc
 -700 ttctagagtg atctctctga gcactactga atacatctaa gacatgtgcc
 -650 tcttgaatat gcttagacaa ttacctgtag gatataatta ggggttaacat
 -600 tttaattctt gcttctctct tttaactcat ggatcttaat tattttattc
 -550 tttaggtgca tgggagttag ttgatgagaa ctctcaaaat cgttacacat
 -500 aatgggttgg ttgaaccac attcttttat cagtatgac agaagatgga
 -450 actcttcata agcattgtac ataagtaggg ggtgaacat gtactctcct
 -400 actcttttga tatataggtg gtcacacttg aattacccat gacacagtgt
 -350 tagtttatag tccataattt tttttcctaa tttagagaac ataagattta
 -300 ccttttagaa attcttgatg aaaatagatt ttaggaggta gatgaacat
 -250 cttagattaat tttagataat taaaatccca ctataaaatt **aaaatggtat**
 -200 tgggtgtctg ggcaaggact tggattcttt ctagaaggac ggcacaaaaa
 -150 taatgagatg gataaatttc atccatccat gattttattca gctagtcaac
 -100 caagtttgaa caaagaaaa gagccccgcg **tgacacgtct** accccctcac
 -50 ctgcctactc aaaccactc **t atataa**ggaa cccaaccctc tctctcatcc
 +1 atcccacaaa cagtttagta ggaagtccaa acaaaacccc ggccggaacg
 +51 **ATG**

Fig. 6. The promoter sequence of lily *PR10g* gene. Numbering starts at the putative transcription start site A. The non-coding sequence is in lower case and the coding sequence (i.e. ATG) is in upper case. The putative transcription start site, putative TATA box and anther/pollen-specific GAATTGTGA, GTGA and AGAAA sequences, which are similar to the conserved sequence motifs in the promoter region of some other pollen-specific genes, are underlined and in bold type.

from -1183 bp to -880 bp upstream of the putative transcription start site is a decisive regulatory region required for the anther-specificity expression of *PR10g* during anther development.

Table 1

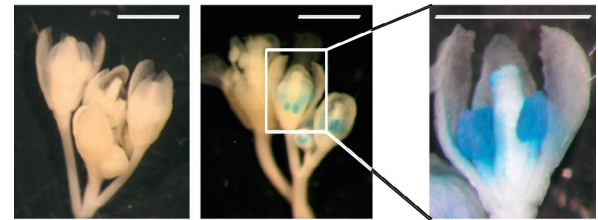
Analysis of putative regulatory *cis*-elements present in the *PR10g* promoter using the PLACE database.

Putative <i>cis</i> -element	Function	Location
TTTTTTC	GA induction	-324
CCTTTT	GA response; Sugar repression	-295, -1132
CATGCA	ABA response	-818
ACACN ^a N ^a G	ABA response	-370
CACATG	Dehydration-responsive; ABA signaling	-655, -820
W ^b AACA	Dehydration-responsive	-85, -953
GTTAGTT	Ethylene response; disease resistance	-346, -529
TGTC	Disease resistance	-56, -357, -377, -708, -1028
GTGA	Pollen-specific expression	-68, -690, -921
AGAAA	Pollen-specific expression	-83, -290
TAAATGTGA	Anther/pollen-specific expression	-202
GCCAC	Light induction	-1129
TTGAC	WRKY DNA-binding site	-102, -1029

^a N = A/T/G/C.

^b W = A/T.

A



WT

PR10gPro::GUS

B

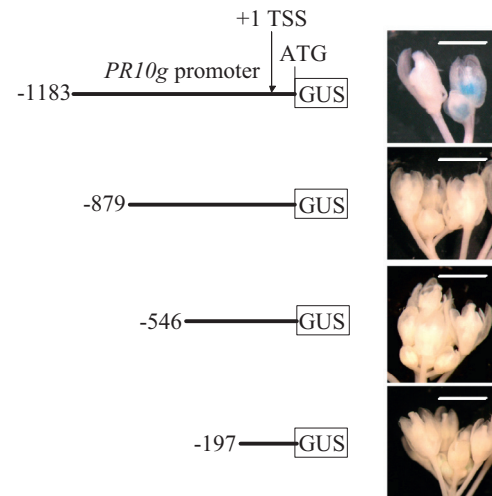


Fig. 7. The specificity of lily *PR10g* promoter in transgenic *Arabidopsis*. (A) The buds of wild-type (WT) *Arabidopsis* and *PR10gPro::GUS* transgenic plants were used for GUS histochemical staining. (B) Deletion analysis of the *PR10gPro::GUS* constructs. The left panel showed a schematic map of the promoter deletion constructs. The relative position of the putative transcription start site (TSS) and translation start codon (ATG) is indicated. The right panel showed the corresponding GUS histochemical staining of transgenic buds. Bar = 2 mm.

3.7. Male sterility in *PR10gPro::barnase* transgenic lines caused by anther developmental arrest

The 1183 bp *PR10g* fragment was fused with *barnase*, a cytotoxic ribonuclease gene from *Bacillus amyloliquefaciens*, and introduced into *Arabidopsis* to further examine the specificity of the tapetum/microspore-specific promoter. If *PR10g* is an anther-specific promoter, the anther growth in the transgenic lines could be arrested due to the production of barnase in the tapetum/microspore of the anther. More than 10 transgenic lines (T1) with the insertion of *barnase* gene were obtained, two of which were chosen for detailed analysis. As shown in Fig. 8A, the *PR10gPro::barnase* transgenic anthers contain few pollen whereas wild-type pollen develops normally. The *PR10gPro::barnase* transgenic lines grew normally, but their flowers had shorter filaments and siliques compared with those of the wild-type plants (Fig. 8A and B). When the female organs of *PR10gPro::barnase* transgenic lines were cross-pollinated with wild-type pollen (indicated by arrowheads), the resulting *PR10gPro::barnase* transgenic siliques reverted to the full size of the wild-type siliques (Fig. 8B, right panel) with normal amounts of well developed seeds, indicating that the promoter was not active in female gametophytes and embryos.

The cross-sections of the *PR10gPro::barnase* anther were further examined at various developmental stages. Sanders et al. [37] divided anther development into 15 stages based on the occurrence of major events during the development. The microspore mother cells at Stage 7 underwent meiosis within the locule and

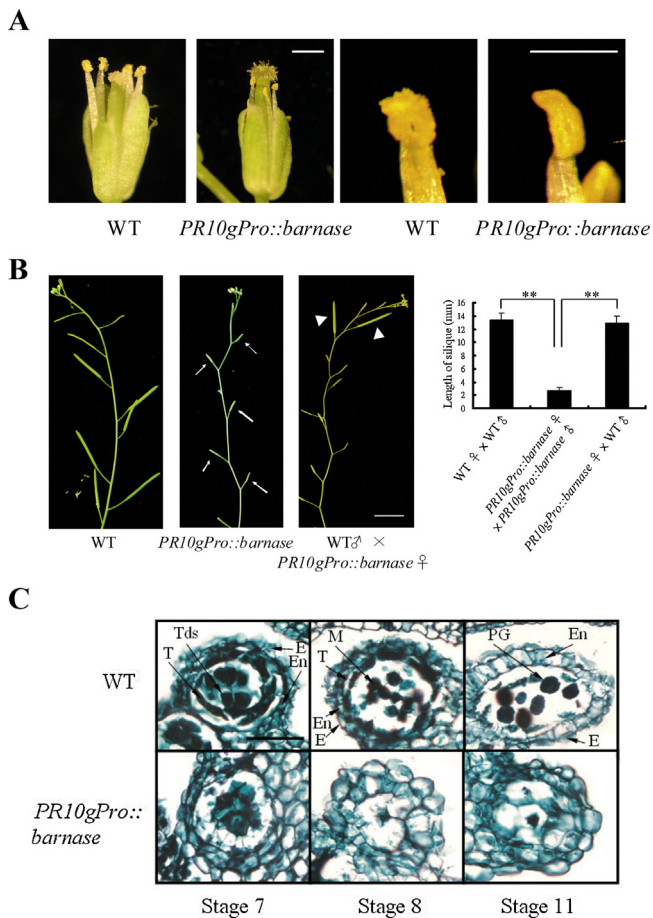


Fig. 8. Phenotypes of wild-type *Arabidopsis* and *PR10gPro::barnase* transgenic plants. (A) The flower and anther of wild-type (WT) *Arabidopsis* and *PR10gPro::barnase* transgenic plants were photographed. The *PR10gPro::barnase* transgenic plants had shorter filaments than WT. Bars=0.5 mm. (B) The inflorescence and siliques of WT *Arabidopsis*, *PR10gPro::barnase* transgenic plants and transgenic plants pollinated with WT pollen were photographed (left panel). Siliques (arrows) of *PR10gPro::barnase* transgenic lines were markedly shorter than those of WT. However, the dwarf siliques were rescued when the female organs of *PR10gPro::barnase* were pollinated with WT pollen (arrowheads). Bar = 10 mm. The length of WT, *PR10gPro::barnase* or *PR10gPro::barnase* female organs pollinated with WT pollen was measured (right panel). The data were obtained from three independent experiments. Around 30 siliques of each sample were measured for each experiment. Error bars represent SD (*t*-test: ** $P < 0.01$). (C) Transverse sections of paraffin-embedded anthers of wild-type and *PR10gPro::barnase* transgenic plants at different developmental stages. The transverse sections of the anther were stained with toluidine blue and photographed by bright-field microscopy. The definition for stages of *Arabidopsis* anther development is based on Sanders et al. [37]. E, epidermis; En, endothecium; M, middle layer; MMC, microspore mother cell; MS, microspore; PG, pollen grains; T, tapetum; Tds, tetrads. Bar = 25 μ m.

generated tetrads of the haploid microspores. Concomitant with the occurrence of meiosis, the tapetal tissue became densely cytoplasmic and polarized such that the vacuole was directed toward the outside and the cytoplasm was directed toward the locular side. However, the anther lobe of the *PR10gPro::barnase* plants had abnormal tetrads crowded into a mass, and the development of the tapetal cells was arrested (Fig. 8C). Subsequently, the microspores were released from the tetrads at Stage 8. This event coincided with the degeneration of the tapetum in wild-type anthers. However, the microspores drastically degraded in *PR10gPro::barnase* anthers, and the cell layers became disorganized. At Stage 11, the endothecium of the wild-type anther wall layers expanded and the tapetum further disintegrated, leaving its remnants in the locule. Pollen mitotic division occurred within the locule, and premature pollen grains formed. However, the anther cell layers of the *PR10gPro::barnase*

lines remained disorganized, and no regular pollen grain formed in the anther (Fig. 8C). The development of the *PR10gPro::barnase* anthers was affected greatly during the three stages of anther development. The effects were especially noticeable during the disruption of tapetum and the deformation of microspore/pollen that resulted in the male sterility of *PR10gPro::barnase* transgenic lines.

4. Discussion

The lily *PR10* genes are anther specific [5]. Herein, we have further demonstrated that *PR10* genes are expressed exclusively in the tapetum and microspores and is modulated by a crosstalk between GA and ethylene during anther/pollen development. The tapetum/microspore specificity of lily *PR10g* is determined both in lily anthers and in transgenic *Arabidopsis*.

The lily *PR10* genes are expressed in the tapetum, as well as coordinately expressed in the microspores during anther development (Figs. 1 and 2). The temporal expression pattern of *PR10* was correlated with tapetal growth/degeneration and microspore formation. Gametophytic microspores are structurally distinct from the tapetum, which is of sporophytic origin in the anther. The coordinate expression of lily *PR10* both in the tapetum and microspores is consistent with the concept that tapetum is the key sporophytic tissue controlling microspore development [38,39]. Aside from *PR10*, we have identified a number of lily genes coexpressed in tapetum and microspores [29,40,41]. An anther-specific cysteine-rich protein in tomato and a chalcone-synthase-like gene in tobacco also follow this expression pattern [42,43]. Very few identified promoters are active at the microspore stage during anther development [38,44]. The lily *PR10g* promoter is tapetum/microspore-specific, similar to the *BCP1* promoter from *Brassica campestris* [45] and different from the tobacco *NTM19* promoter, which exhibits strict microspore-specific expression with no activity in mature pollen [38].

The development of tapetum and microspores is regulated by plant hormones [46]. GAs are synthesized in the tapetum and play a critical role in tapetum development [31,47]. We have identified a set of GA-regulated genes in the tapetum [34]. As a GA-regulated gene, lily *PR10* is upmodulated by endogenous GA during anther development and it is also induced by the exogenous addition of GA. Both uniconazole and NBD may seriously affect anther development. Treatment with both inhibitors decrease the *PR10* gene expression level compared with the one treated with only NBD. Thus, an antagonistic interaction between GA and ethylene likely modulates *PR10* gene expression in young anthers although the level of cross-talk is still unclear. Consistent with *PR10* induction through crosstalk between GA and ethylene, the *PR10g* promoter region contains *cis*-elements for GA induction (TTTTTCC and CCTTTT) and for ethylene response (GTTAGTT) [48–50]. Few reports have demonstrated the involvement of GA and ethylene in tapetum/anther development [46,51]. It should be noted that the antagonistic effect of ethylene on the GA activity occurs not only in the anther but also in other parts of floral organs. GA reportedly promotes cell elongation in both filament and corolla segments, while ethylene inhibits growth [52]. Various hormones and environmental signals in plants form a sophisticated network of signal transduction pathways [33]. Multiple cross-talks may occur between GA and ethylene. Whether this cross-talk is additive, synergistic or antagonistic is dependent on the process and on the developmental stage in *Arabidopsis thaliana* [51]. The tapetum is the source of GAs in the flower [31], and the identification of GA-regulated genes is an important step toward understanding the role of gibberellin and its interactions with other hormones in the anther during development.

Examination of the 5'-regulatory region of *PR10g* reveals the presence of various potential *cis*-elements related to pollen/anther-specific regulatory motifs such as TAAAATGTTA, AGAAA, and core GTGA motifs (Table 1). These *cis*-elements are also conserved in the promoter region of a rice anther-specific *RTS* gene [53] and three tomato pollen-specific *LAT52*, *LAT56* and *LAT59* genes [35,36]. Therefore, the similarities of the regulatory regions of *PR10g* and other anther/pollen-specific genes among monocot and dicot plants suggest that some basic features governing gene expression in the anther are conserved, leading to the preservation of anther/pollen-specific expression of the lily *PR10g* promoter when introduced into dicot species, such as *Arabidopsis* plants. A number of rice anther/pollen genes and their promoters are able to drive anther-specific gene expression in monocot and dicot plants [53–55]. Given that lily transformation is still unavailable, the lily *PR10g* promoter was analyzed using *Arabidopsis* as a model system. When the *GUS* gene was driven by the full-length *PR10g* promoter, its specific expression was observed in the young anthers of the transgenic plants (Fig. 7). The promoter-driven *GUS* activity was strongly and specifically expressed in young anthers, but not in the anthers of mature flowers and not in any vegetative organ of the *PR10gPro::GUS* transgenic *Arabidopsis* lines. Furthermore, the *PR10g* promoter fragment from -1183 bp to -880 bp upstream of the putative transcription start site is determined to be a decisive regulatory region required for the anther-specific expression of *PR10g* during anther development. This fragment contains a core GTGA motif that has been demonstrated to be crucial in directing pollen-specific gene expression [35,36]. Accordingly, the core motif may possibly be important in directing microspore-specific gene expression. The tapetum/microspore-specific promoter of lily *PR10g* is further reinforced by the production of male-sterile *PR10gPro::barnase* transgenic lines, wherein tapetum and microspore development are badly disrupted and deformed (Fig. 8C). Therefore, the lily *PR10g* promoter retains its specificity and functions in directing tapetum/microspore-specific gene expression in lily as well as in *Arabidopsis*. The unique feature of anther-specificity differs from that of the pine *PR10* promoter, in which promoter-driven *GUS* activity occurs in various plant vegetative organs [12,56].

When the lily *PR10g* promoter was fused with the cytotoxic ribonuclease gene, *barnase*, it drives tissue-specific gene expression in *Arabidopsis*, conferring male sterility to the transgenic plants. The development of the *PR10gPro::barnase* anthers was greatly affected during various stages of anther development (Fig. 8C). The defects occurred from abnormal tetrads crowded into a mass during the early stage of anther development into drastically degraded microspores and disorganized cell layers in *PR10gPro::barnase* anthers. Finally, no regular pollen grain formed in *PR10gPro::barnase* anthers. When the female organs of *PR10gPro::barnase* transgenic lines were cross-pollinated with wild-type pollen, the resulting *PR10gPro::barnase* transgenic siliques reverted to the full sized the wild-type siliques with normal numbers of well developed seeds (Fig. 8B). This means that the *PR10g* promoter was completely silent during female gametogenesis and embryogenesis. The use of the cytotoxic *barnase* reporter gene rules out the possibility of *PR10g* promoter expression in the endosperm, as was found for the late pollen-specific *LAT52* and *LAT59* promoters [57].

Luo et al. [53] have demonstrated the availability of the rice *RTS* gene as a useful tool for controlling gene expression in anthers of various plant species. Similar to *RTS*, the lily *PR10* is capable of directing tapetum-specific gene expression in lily plants. Aside from being tapetum-specific, the lily *PR10* gene is microspore-specific. The *PR10g* promoter-driven *GUS* activity was specifically and strongly expressed in young anthers, but not in the anthers of mature flowers and not in the vegetative organs of the

transgenic *Arabidopsis* lines. Thus, the *PR10g* promoter could be an invaluable tool for directing high levels of transgene expression to the microspores during culture to study of biological mechanism by which microspores change their fate toward embryogenesis [38]. In addition, the microspore-specific promoters can be used for further molecular dissection of early pollen development [38] and for engineering transgenic plants to produce pollen devoid of any transgene [58].

In summary, the lily *PR10g* genes are modulated specifically and differentially in the tapetum and the microspores of young anthers through the antagonistic crosstalk between GA and ethylene during development. The anther specificity of the lily *PR10g* promoter highlights the importance of the tapetum/microspore-specific promoter for future biotechnological and agricultural applications.

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