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Expression and localization of two anther-specific genes in the tapetum and microspore of *Lilium longiflorum*

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ABSTRACT. Two stage-specific genes have been isolated from a subtractive cDNA library constructed from developing anthers of *Lilium longiflorum*. Tandem 5′- and 3′-rapid amplifications of cDNA ends with polymerase chain reaction (5′- and 3′-RACE-PCR) were used to obtain the full length cDNA sequences. The *LLA-89* cDNA contained an open reading frame of 303 bp encoding an acidic polypeptide of 100 amino acids with a calculated molecular mass of 10.2 kDa. The *LLA-142* cDNA contained an open reading frame of 171 bp encoding a basic polypeptide of 56 amino acids with a calculated molecular mass of 5.7 kDa. The protein encoded by *LLA-89* had a strong hydrophobic region at the N-terminus, indicating the presence of a signal peptide (not found in the LLA-142 protein). Sequence alignment revealed that the protein encoded by *LLA-142* is novel, while the protein encoded by *LLA-89* is identical to a reported LIM4 protein with unknown function. Total RNA blot analysis indicated that the transcripts of *LLA-89* and *LLA-142* were anther-specific and differentially detected in the anther wall and in the microspore of developing anthers. The *LLA-89* gene could be exogenously induced by gibberellin while the *LLA-142* gene could not. *In situ* hybridization with antisense riboprobes for the two genes in the anther revealed strong signals localized to the tapetal layer of the anther wall. The function of *LLA-89* and *LLA-142* genes was further discussed.

Keywords: Anther-specific gene; Gibberellin; *Lilium longiflorum*; Microspore; Tapetum.

Abbreviations: DIG, digoxigenin; **GA**, gibberellin; **LLA**, *Lilium longiflorum* anther; **PCR**, polymerase chain reaction; **RACE**, rapid amplification of cDNA ends.

INTRODUCTION

In higher plants, male gametophyte (pollen) formation occurs in a specialized floral organ, the stamen. The stamen is the male reproductive organ that consists of anthers and filaments. The young anther is composed primarily of sporogenous tissue surrounded by wall layers. The sporogenous cells undergo meiosis and give rise to microspores enclosed by callase at the tetrad stage. Subsequently, the enclosed microspores are released into the locule of anthers by callose that is synthesized and secreted from the tapetum. The microspores further develop into pollen after haploid mitosis and further differentiate into mature and dried pollen (McCormick, 1993; Scott et al., 2004).

The tapetum, an innermost layer of the anther wall, represents a secretory tissue that provides nourishment for microspore development (Ma, 2005). The tapetum synthesizes and secretes enzymes and sporopollenin precursors that contribute to the completion of pollen grain wall development (Huang et al., 2009; Li et al., 2010). Moreover, the tapetal cells provide pollen with adhesive and signaling molecules that are critical for pistil interaction during pollination (Suen et al., 2003). Without a differentiated tapetum, critical nutrient resources are not available for proper microspore/pollen differentiation and maturation (Kapoor et al., 2002; Li et al., 2006; Shi et al., 2009).

Anther/pollen development is controlled primarily by two broad classes of early and late genes. Early gene expression occurs first in microspores but is reduced in mature pollen. Concomitant with the early gene expression in the microspore, a large number of early genes are expressed in tapetal cells (Ma, 2005). These genes might encode proteins needed for cell wall synthesis, starch deposition, and microspore growth. To understand the function of tapetum at the molecular level, several research groups have identified tapetum-specific transcripts from various species (Chen et al., 2006; Grienenberger et al., 2009; Ishiguro et al., 2010; McNeil and Smith, 2010). Gibberellin (GA) is reportedly synthesized in the tapetum (Kaneko et al., 2003), and tapetal genes regulated by GA and other hormones have been documented (Van den Heuvel et al., 2002; De Grauwe et al., 2007; Hirano et al.,

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2008; Tzeng et al., 2009). We constructed a subtractive cDNA library for lily anthers at the microspore stage and from it we identified numerous anther-specific genes (Hsu et al., 2008). Of these genes, *LLA* (*Lilium longiflorum anther*) *-89* and *LLA-142* were chosen for further investigation. The two gene transcripts accumulated specifically at the stage of anther microspore development. The *LLA-89* gene could be GA-induced but the *LLA-142* gene could not. Gene expression analyses by *in situ* hybridization and RNA blotting detected mRNAs in the microspore and tapetum of these two genes.

MATERIALS AND METHODS

Plant material

Lily plants (*Lilium longiflorum* Thunb. cv. Snow Queen) were grown in the field. The anthers dissected from 10-170 mm buds ranged from 7-23 mm in length. Meiosis occurred in the pollen mother cells when buds were around 20-25 mm, resulting in the formation of tetrads. After completion of the first microspore mitosis in the 65-70 mm buds, immature pollen formed and later matured and dried. Concomitant with the microspore development, the anther wall tapetum became secretory, after which it degenerated. The anthers and filaments were collected separately in buds longer than 25 mm, but were combined and analyzed together. The first three arrays of young leaves around buds, entire roots (approximately 8 cm from the apex), stems and individual reproductive organs were dissected and immediately frozen in liquid nitrogen. All materials were stored at -80°C until use. For the duration of the GA_3 (Sigma-Aldrich, St. Louis, MO, USA) treatment, lily plants with varying bud size were cut around 150 mm from the top and dipped in aqueous solutions containing various concentrations of GA_3 for a period of 3-36 h.

Plasmid isolation and sequence analysis

The *LLA-89* and *LLA-142* cDNA clones were identified from a subtractive cDNA library at the stage of lily anther microspore development (Hsu et al., 2008). Plasmid DNA was purified from *LLA-89* and *LLA-142* cDNA clones. The cDNA cloned in the pGEM-T Easy vector (Promega, Madison, WI, USA) was digested with *Rsa*I to determine the insert size. 5′- and 3′- rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR) was done according to the SMARTTM RACE cDNA amplification kit user manual (CLONTECH Laboratories, Inc., CA, USA). DNA sequences from both strands of the cloned inserts were obtained using an ABI Model 377 automated sequencer (Foster City, CA, USA). Sequence alignment was achieved using the Vector NTI Suite 8 software (InforMax, Inc., Bethesda, MD, USA), and the homology search was done using BLAST (Altschul et al., 1997).

RNA blot

Total RNA was extracted from developing anthers and from other floral and vegetative organs using the Ultraspec RNA isolation system (Biotecx Laboratories, Inc.). To separate microspores from the anther wall, anthers of young buds were transversely sliced with a scalpel, after which microspores were gently squeezed out and into a 10 mM sodium acetate, 5.2 pH buffer. After 3 min centrifugation at 5,000 x*g*, the pellet (microspores) was ready for total RNA extraction. RNA samples were electrophoresed in 1.0% formaldehyde-3-[*N*-morpholino]-propanesulfonic acid gels using standard procedures (Sambrook et al., 1989) and transferred onto nylon membranes (Micron Separation, Inc.). The membranes were prehybridized for 4 h at 42°C in medium containing 5x SSC (750 mM 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) sodium dodecyl sulfate (SDS), 1% glycine, 50% formamide and 150 μg/mL of denatured salmon sperm DNA. For hybridization, the prehybridization solution was removed and replaced with hybridization buffer that contained the same components as the prehybridization buffer but with 1% glycine. Denatured salmon sperm DNA (100 μg/mL), random primed ^{32}P-labeled probe (1.0×10^9 cpm/μg) of *LLA-89* and *LLA-142* cDNAs were respectively added to the hybridization buffer. Hybridization was carried out at 42°C overnight under constant agitation. The membranes were washed at 42°C in 2x SSC, 0.1% (w/v) SDS twice for 20 min, and at 65°C in 0.1x SSC, 0.1% (w/v) SDS twice for 20 min. The membrane was either visualized using phosphoimager plates or exposed to X-ray film (Konica AX) using one or two intensifying screens (DuPont) for 3 d or less. At least two independent experiments were carried out for the RNA blot analysis.

RNA *in situ* hybridization

Anthers dissected from lily buds of various sizes were fixed at 4°C for 16 h under vacuum in 1x 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, dehydrated and then embedded in Steedman's wax. The embedded tissues were sectioned (7-10 μm) with a microtome and affixed to poly-L-Lys-coated slides. DIG (digoxigenin)-labeled RNA probes were synthesized using a DIG RNA labeling kit (SP6/T7) (Roche Diagnostics GmbH, Penzberg, Germany). *In situ* hybridization was carried out generally following the DNA *in situ* hybridization protocol previously described in Kao et al. (2006) with some modifications. Hybridization sites were immunologically detected using an alkaline phosphatase conjugated anti-DIG antibody diluted 1:500 with 1x 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 for 2 h with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates of alkaline phosphatase. The hybridization signal viewed under a bright-field microscope was brownish purple. Sections were counterstained with 0.001% Fast Green.

RESULTS

Characterization of the two stage-specific cDNAs in the anther of *L. longiflorum*

The *LLA-89* and *LLA-142* cDNAs were identified from a subtractive cDNA library at the *L. longiflorum* anther microspore development phase (Hsu et al., 2008). Since the insert sizes of both cDNAs were partial, the 5′- and 3′-RACE-PCR method was used to obtain the full length 493 bp *LLA-89* cDNA (accession no. DQ907930) and the full length 379 bp *LLA-142* cDNA (accession no. EF026007), both excluding the poly (A) tail.

The *LLA-89* cDNA contains an open reading frame of 303 bp encoding a polypeptide of 100 amino acids with a calculated molecular mass of 10.2 kDa and a pI of 3.2 (Figure 1A). The *LLA-89* cDNA insert is close to full-

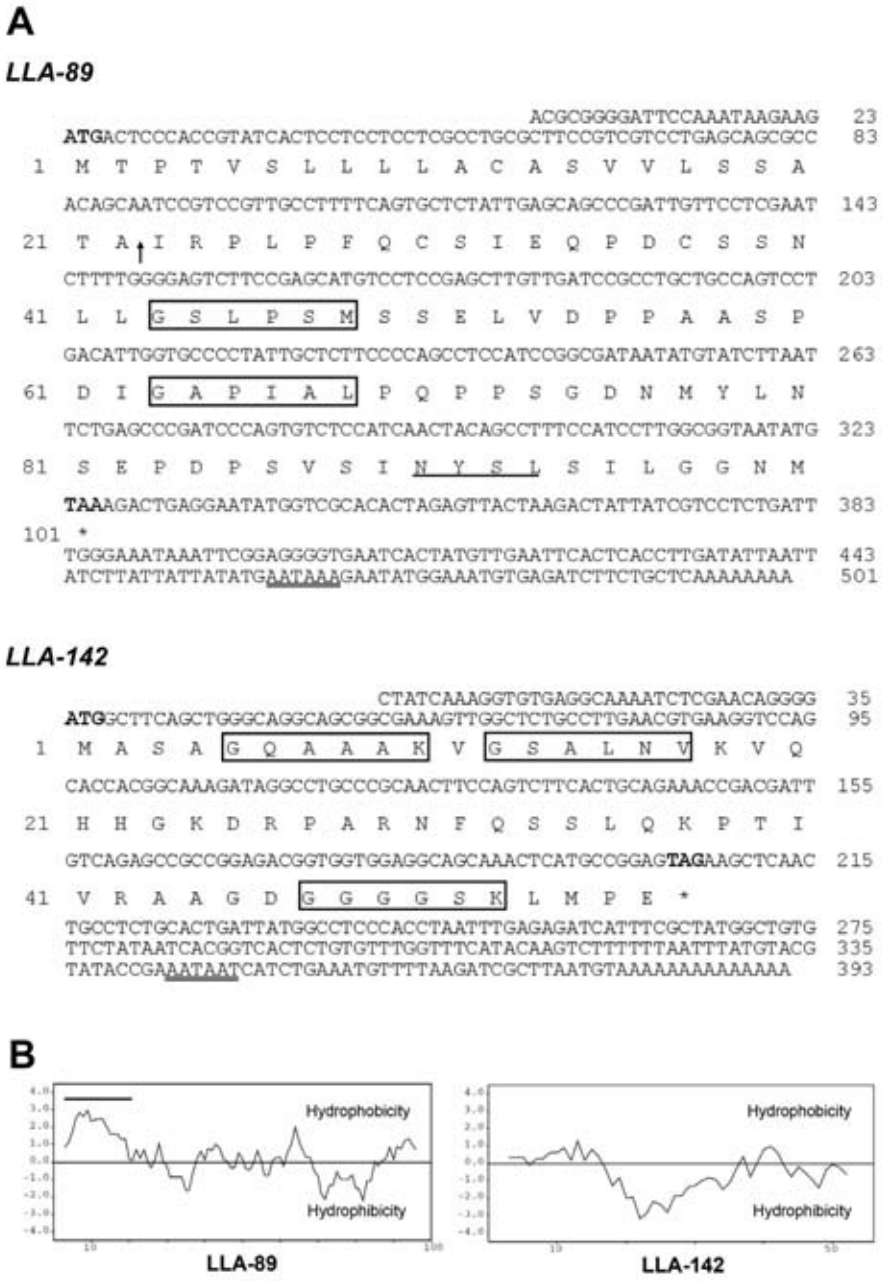

Figure 1. Sequences and hydropathy profiles of LLA-89 and LLA-142 in *L. longiflorum* anther. (A) Nucleotide and predicted amino acid sequences of *LLA-89* and *LLA-142* cDNAs. Numbers of nucleotide and amino acid sequences are indicated on the right and left, respectively. Bold letters in the nucleotide sequence indicate start codon and stop codon. The arrow indicates the predicted cleavage site of the signal peptide. The double underlines indicate the putative polyadenylation signal site. The underline indicates the putative N-glycosylation site (N-X-S/T). The putative N-myristoylation sites (G-X-X-X-S/T/A/G/C/N) are boxed; (B) Hydropathy profiles (Kyte and Doolittle, 1982) of LLA-89 and LLA-142 protein sequences. The black line indicates a hydrophobic sequence at the N-terminus of the sequence.

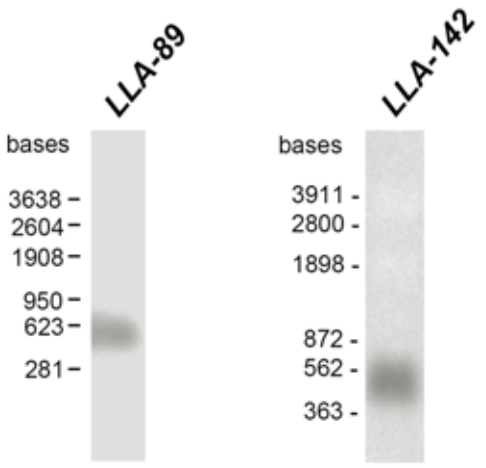

Figure 2. An estimation of the full-length of *LLA-89* and *LLA-142* mRNAs in the anther of *L. longiflorum*. Total RNA (20 µg) was isolated from anthers within 34-36 mm-long buds. Total RNA was denatured, fractionated on formaldehyde-agarose gels, transferred onto nylon membranes and hybridized with the ^{32}P-labeled *LLA-89* and *LLA-142* cDNA inserts. The RNA ladder markers are indicated at left.

length compared with its mRNA visualized using RNA blot analysis (Figure 2). In the 3´-untranslated region, a putative AATAAA consensus motif for polyadenylation signals is located 34 bp upstream from the site of polyadenylation. A putative N-glycosylation site (N-X-S/T) is present at the amino acid position 90. Two putative N-myristoylation sites (G-X-X-X-S/T/A/G/C/N) are present at the amino acid positions 43 and 63. Assessment of the hydropathy profile (Kyte and Doolittle, 1982) showed that the LLA-89 polypeptide has a strong hydrophobic region near the N-terminus, indicating the presence of a signal peptide (Figure 1B). The proposed cleavage site in LLA-89 is between positions 22 and 23 (Figure 1A). The predicted amino acid sequence of *LLA-89* was utilized to search protein databases. Sequence alignment revealed that LLA-89 is identical to LIM4, a protein known to be induced during the early prophase of meiosis in microsporogenesis of *L. longiflorum* (Kobayashi et al., 1994).

The *LLA-142* cDNA contains an open reading frame of 171 bp encoding a polypeptide of 56 amino acids (Figure 1A) with a calculated molecular mass of 5.7 kDa and a pI of 11.1. The *LLA-142* cDNA insert is close to full-length compared with its mRNA, visualized using RNA blot analysis (Figure 2). In the 3´-untranslated region, a variant AATAAT consensus motif of polyadenylation is located 36 bp upstream from the site of polyadenylation. Three putative N-myristoylation sites are present at amino acid positions 5, 12, and 47. While the LLA-89 polypeptide contains a signal peptide at the N-terminus, the LLA-142 polypeptide, as shown by hydropathy profile analysis, does not (Kyte and Doolittle, 1982) (Figure 1B). The predicted amino acid sequence of LLA-142 was utilized to search protein databases. Sequence alignment revealed no significant resemblance to known proteins, indicating that *LLA-142* encodes a novel protein.

Temporal and spatial expression of the two anther-specific genes in *L. longiflorum*

To determine the expression patterns of the two genes during anther development, blots of total RNA isolated from anthers at different stages, based on lily bud size, were hybridized with ^{32}P-labeled *LLA-89* and *LLA-142* cDNAs. These two genes were differentially expressed during anther development. The *LLA-89* transcripts were first detected in anthers within 20-25 mm-long buds, a stage of anther development concomitant with meiosis of the pollen mother cell. The *LLA-89* mRNA accumulated to reach a maximum level in the anther within 34-36 mm-long buds, corresponding to the phase of microspore development and major cytological changes in the wall

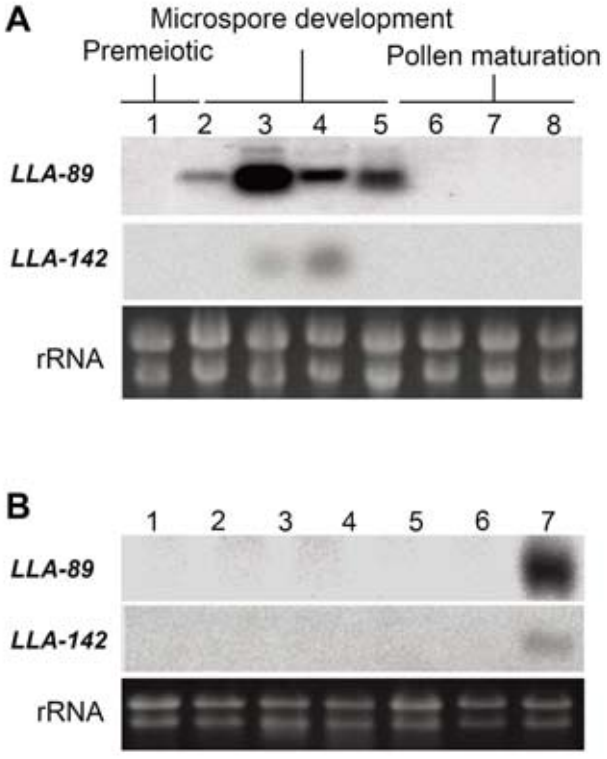

Figure 3. Temporal and organ-specific expression of *LLA-89* and *LLA-142* genes in the *L. longiflorum* anther. Total RNA (20 µg) was isolated (A) from stamen/anthers of different size classes of buds: 1 ≤ 15 mm buds; 2 = 20-25 mm buds; 3 = 34-36 mm buds; 4 = 44-46 mm buds; 5 = 60-65 mm buds; 6 = 90-95 mm buds; 7= 120-125 mm buds; 8 = 150-155 mm buds, and (B) from various vegetative and floral organs: roots (lane 1), stems (lane 2), leaves (lane 3), tepals (lane 4), carpels (lane 5), filaments (lane 6) and anthers (lane 7) within 34-36 mm-long buds. Total RNA was denatured, fractionated on formaldehyde-agarose gels, transferred onto nylon membranes, and hybridized with the ^{32}P-labeled *LLA-89* and *LLA-142* cDNA inserts. Almost equal amounts of total RNA were loaded in each lane, as determined by ethidium bromide staining of the gel.

layers. The *LLA-89* mRNA amount decreased significantly but retained its signal in the 60-65 mm-long bud anthers, during which time the first mitosis occurred in the microspore to form immature pollen. The *LLA-142* transcripts, however, were mainly restricted to the anther microspore development phase (Figure 3A). Signals were detected neither in the premeiotic phase nor in the pollen maturation phase. Overall, both *LLA-89* and *LLA-142* transcripts were detected during the microspore development phase.

To determine the tissue-specificity of gene expression in lily plants, total RNA was isolated from vegetative organs (roots, stems, and leaves) and floral organs within the 34-46 mm-long buds (tepals, filaments, anthers, and carpels comprising stigmas, styles and ovaries). The isolated mRNA on a blot was hybridized with ^{32}P-labeled *LLA-89* and *LLA-142* cDNAs (Figure 3B). With the exception of the anther, no hybridization signal was detected in RNA samples from other lily organs, indicating that the two genes were anther-specific.

Effects of GA on the regulation of the two anther-specific genes in developing anthers of *L. longiflorum*

To investigate the inductive effect of GA on gene expression, we applied GA exogenously. Lily plants with 17-20 mm-long buds were dipped in solutions containing various concentrations of GA_3 for 24 h. Total RNA blot analysis revealed that the *LLA-89* gene was induced by as little as 1 µM GA_3. In contrast, the *LLA-142* gene did not respond to GA_3 (Figure 4A). The *LLA-89* mRNA accumulation level increased as the GA_3 concentration increased.

In order to determine the kinetics of the GA_3-stimulated accumulation of *LLA-89* mRNAs in lily anthers, a time-course experiment was conducted. Lily plants with 17-20 mm-long buds were dipped in a solution containing 1 µM GA_3 for various periods of time. Blot analysis showed that the *LLA-89* mRNA started to accumulate after 18 h of GA_3 treatment (Figure 4B, lane 3) and peaked at 36 h post-treatment (lane 4). As a control, no signal was detected in the anther of lily buds 36 h after treatment without the addition of GA_3 (lane 5).

In situ localization of the two anther-specific mRNAs in developing anthers of *L. longiflorum*

In situ hybridization the two genes with DIG-labeled antisense RNA probes was performed to determine the cellular location of these transcripts. No positive signal was detected in sections of anthers from 35-40 mm-long buds treated with sense probes (Figure 5A and B). When the DIG-labeled antisense riboprobes of *LLA-89* and *LLA-142* were used, hybridization signals with dark brownish-purple signals were observed in the tapetum of 35-40 mm-long buds (Figure 5C-F). At this time, the tapetal cells became polarized and highly secretory in the anther. The brownish-purple signals observed in the tapetum indicated that transcripts of the two genes were significantly expressed in the tapetum when compared to other anther wall layers. However, hybridization signals in the microspores were not discernible due to the structure distortion. As buds grew to 45-50 mm, the tapetal cells in the anther began to disintegrate (Figure 5G-J) and the hybridization signal strength decreased compared to that observed in the tapetum of the 35-40 mm-long buds, suggesting significant down-expression of the *LLA-89* and *LLA-142* genes (Figure 5G-J).

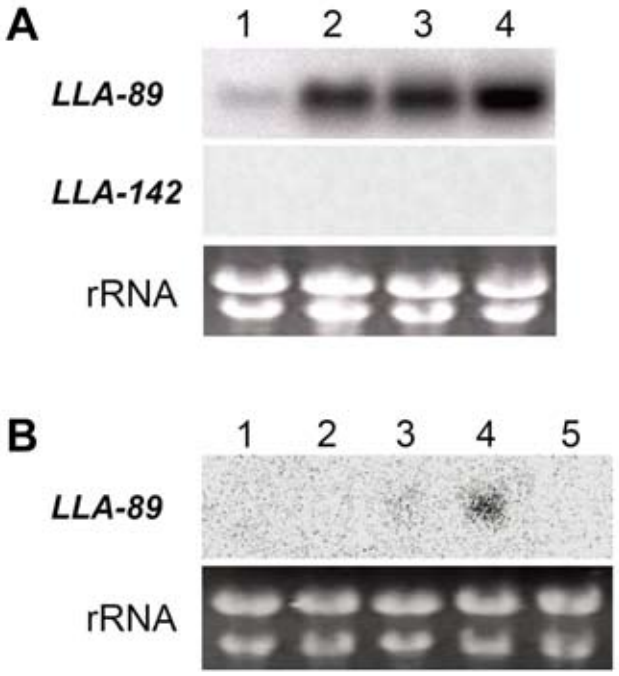

Figure 4. Transcript induction of *LLA-89* and *LLA-142* genes by GA_3. Total RNA (20 µg) was isolated from anthers (lane 1) within 17-20 mm-long buds. (A) The buds were dissected from lily plants, dipped in solutions containing 1 µM (lane 2), 10 µM (lane 3), and 100 µM (lane 4) GA_3 for 24 h. (B) The buds were dissected from lily plants dipped in a solution containing 1 µM GA_3 for 3 h (lane 1), 9 h (lane 2), 18 h (lane 3), 36 h (lane 4), and without GA_3 addition for 36 h (lane 5). Total RNA was denatured, fractionated on formaldehyde-agarose gels, transferred onto nylon membranes and hybridized with the ^{32}P-labeled *LLA-89* and *LLA-142* cDNA inserts. Almost equal amounts of total RNA were loaded in each lane, as determined by ethidium bromide staining of the gel.

Accumulation of the two gene transcripts in the developing microspores of *L. longiflorum*

To verify whether the two genes were expressed in the microspore, anthers of various size classes were dissected into two parts: the anther wall and the microspores. A blot of total RNA isolated from microspores of various developmental stages was hybridized with ^{32}P-labeled *LLA-89* and *LLA-142* cDNAs. In addition to detection in the anther wall (tapetum), both *LLA-89* and *LLA-142* transcripts were coordinately detected in the various stages of microspores in buds up to 44-46 mm (Figure 6 lanes 1-3). No signals

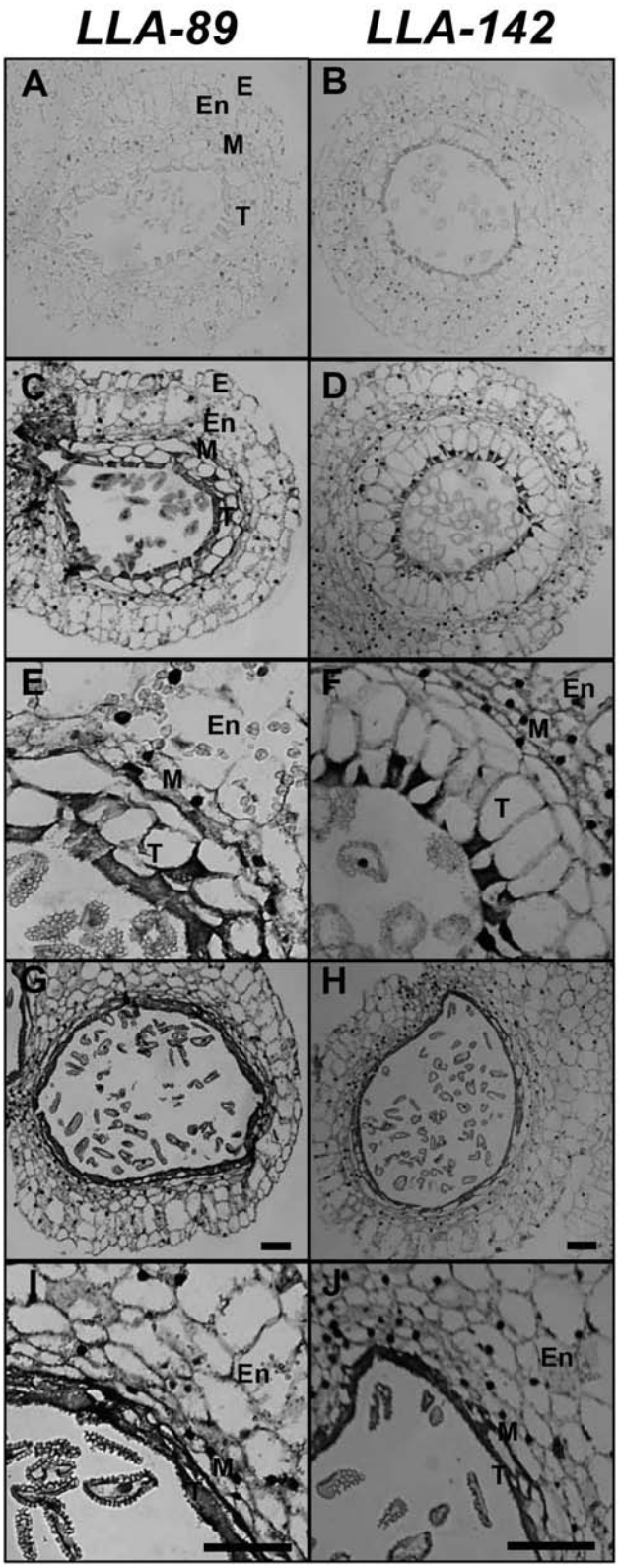

Figure 5. *In situ* hybridization of *LLA-89* and *LLA-142* transcripts in the developing anther of *L. longiflorum.* Expression of *LLA-89* and *LLA-142* was analyzed in bright-field illuminated 7-10 µm cross-sections of anthers within 35-40 mm-long buds (A-F) or 45-50 mm-long buds (G-J) using DIG-labeled sense (A) and antisense (C, E, G, I) *LLA-89* RNA and sense (B) and antisense (D, F, H, J) *LLA-142* RNA probes. E, epidermis; En, endothecium; M, middle layer; T, tapetum. Bar = 100 µM.

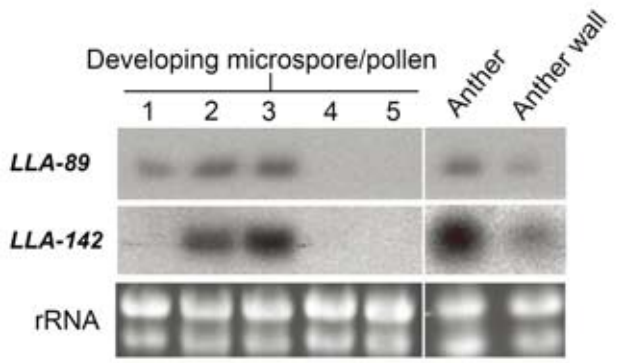

Figure 6. Expression patterns of *LLA-89* and *LLA-142* genes in developing microspores of *L. longiflorum*. Total RNA (20 µg) was isolated from the anther and anther wall within 44-46 mm-long buds and from microspores of various size classes of buds: 1 = 20-25 mm-long buds; 2 = 34-36 mm-long buds; 3= 44-46 mm-long buds; 4 = 60-65 mm-long buds; 5 = 70-75 mm-long buds during the microspore development phase. Total RNA was denatured, fractionated on formaldehyde-agarose gels, transferred onto nylon membranes, and hybridized with the ^{32}P-labeled *LLA-89* and *LLA-142* cDNA inserts. Almost equal amounts of total RNA were loaded in each lane, as determined by ethidium bromide staining of the gel.

were detected from blots of mRNA isolated from the microspore/pollen within 60-65 mm-long buds or thereafter (lanes 4 and 5), further indicating the stage-specificity of *LLA-89* and *LLA-142* gene expression in the anther. The detection of a signal in the anther and anther wall was used as a control.

DISCUSSION

We previously constructed a subtractive cDNA library to identify genes expressed during microspore development in *L. longiflorum*. Of those genes identified only in young anthers, the genes *LLA-89* and *LLA-142* were chosen for further investigation. These two genes were anther-specific and differentially expressed at the microspore development stage (Figure 3). Further, analysis by *in situ* hybridization demonstrated that both gene transcripts accumulated in the tapetum, the innermost layer of the anther wall (Figure 5). In addition to expression in the tapetum, the two genes were coordinately expressed in the microspore at the microspore development phase, as confirmed by microspore RNA blot analysis (Figure 6).

The spatial and temporal expression patterns of *LLA-89* and *LLA-142* correlated with tapetum development and degeneration. Around meiosis, when the buds were 20-25 mm in length, *LLA-89* mRNA began to accumulate, reaching a maximum level when the buds were 34-36 mm-long. During this time, the tapetum became highly secretory in the anther. Afterwards, the *LLA-89* mRNA expression significantly decreased as tapetal cells began to disintegrate (Figures 3A and 5). When compared with *LLA-89*, the level of *LLA-142* mRNA accumulation was more temporally

restricted to a period when the tapetum became highly active and secretory.

The full-length cDNAs of both genes were obtained by 5′- and 3′-RACE-PCR (Figure 1). Both cDNA inserts were close to full-length based on the sizes of their mRNAs demonstrated on the blots (Figure 2). The *LLA-89* gene encodes an acidic polypeptide, whereas the *LLA-142* gene encodes a basic polypeptide. Sequence alignment revealed that *LLA-89* was identical to *LIM4*, a known gene expressed in lily microsporocytes (Kobayashi et al., 1994). It was reported that the *LIM4* transcript was first detected at the zygotene stage of meiotic prophase and continued to be expressed throughout meiosis. Although the mRNA is induced during meiosis, it accumulates to a maximum level during the free microspore development phase prior to microspore mitosis (Figure 6). Therefore, the function of the LLA-89 protein is probably not related to the process of meiosis, as implied by Kobayashi et al. (1994). Furthermore, we found that the gene was not only expressed in the microspore, but also strongly expressed in the tapetum (Figures 5 and 6). The accumulation of *LLA-89* mRNA was associated with major cytological changes in the tapetum (Figures 3 and 5). The LLA-89 protein contained a signal peptide at the N-terminus, so it is possible that the protein, once synthesized in the tapetum and microspores, is secreted and deposited on the walls of pollen grains. Thus, we propose that the LLA-89 protein may be involved in microspore/pollen wall formation. Alternatively, LLA-89 protein may act as a signaling molecule related to pollination or microbial responses based on its small size and the presence of a putative glycosylation and two putative N-myristoylation sites. Protein N-myristoylation plays a vital role in membrane targeting and signal transduction in the plant response to environmental stress (Podell and Gribskov, 2004). It was reported that N-myristoylation of the ZmPtila protein is required for accumulation on the plasma membrane (Herrmann et al., 2006). Ishitani et al. (2000) reported that N-myristoylation was required for the function of the *SOS3* salt tolerance gene product in *Arabidopsis*. Although the function of LLA-89 protein is still not clear, the pattern of expression and structure suggests functions different from meiotic regulation (Kobayashi et al., 1994). Functional investigations of the *LLA-89* gene using genetic approaches in *Arabidopsis* might shed light of the function of LLA-89 protein.

While the LLA-89 protein has a signal peptide at the N-terminus, the LLA-142 protein does not. Sequence alignment revealed no significant resemblance to any known proteins. Since *LLA-142* mRNA is localized to the tapetum, it is reasonable to suggest that the protein remains in the tapetal cells. The tapetum is known for its nutritive role during microspore and pollen development (Ma, 2005; Huang et al., 2009). In addition to a nutritive role, LLA-142 may be involved in one or more of the following developmental events in lily anthers: First, esterases in the tapetum are known to be active during tapetal degeneration (Sawhney and Nave, 1986). Peroxidases and other proteins are associated with the synthesis of sporopollenin or its precursor in the lily tapetum (Reznickova and Willemse, 1980). Finally, proteins are involved in the synthesis of carotenoids and flavonoids that, in turn, form the pollenkitt, a substance deposited on the pollen wall with tapetal debris (Hsieh and Huang, 2007).

We have reported two anther-specific genes in *L. longiflorum*. These two genes were only expressed during the phase of microspore development in the anther and were specifically expressed in the tapetum and microspores. Our analysis indicated that the *LLA-89* gene was induced by the exogenous addition of GA_3, while *LLA-142* was not (Figure 4). In spite of the fact that the *LLA-142* gene was not induced by GA_3, both genes were indeed regulated by GAs in growing anthers as indicated by the observation that the expressions of *LLA-89* and *LLA-142* were partially inhibited by treatment with uniconazole, an inhibitor of GA biosynthesis (Hsu et al., 2008). It is possible that the inconsistency of the *LLA-142* gene response to exogenous GA is due to an insufficient length of GA_3 exposure. Alternatively, although the inductive mechanism of gene activation is not yet clear, it is obvious that the transduction pathway induced by exogenous GA is not the same as that induced by GAs that are naturally produced in the anther. The identification of the two GA-regulated genes is an important step towards understanding the role of GA and its interaction with other hormones in anther development.

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鐵炮百合兩花藥專一基因 *LLA-89* 和 *LLA-142* 之表現和定位

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利用抑制扣除雜合法 (suppression subtractive hybridization) 從鐵炮百合 (*Lilium longiflorum*) 花藥的小孢子發育期 cDNA 集合庫選殖出兩個花藥專一性基因。利用 5′- 與 3′-RACE-PCR 延伸而得到 *LLA-89* 和 *LLA-142* cDNAs 全長序列。*LLA-89* cDNA 含有一段 303 bp 可編譯框架 (open reading frame)，可轉譯出 100 個胺基酸的酸性蛋白質，其分子量爲 10.2 kDa。*LLA-142* cDNA 含有一段 171 bp 可編譯框架可轉譯出 56 個胺基酸的鹼性蛋白質，其分子量爲 5.7 kDa。 LLA-89 蛋白質 N 端有一段疏水性的訊息胜肽 (signal peptide) 序列，而 LLA-142 則無。序列比對後顯示 LLA-89 即是已知的百合 LIM4，而 LLA-142 則是嶄新的未知蛋白質。利用北方墨漬法分析，得知 *LLA-89* 和 *LLA-142* mRNA 均在小孢子發育時期偵測到且具花藥專一性。*LLA-89* 基因會受外加的激勃素 (gibberellin) 誘導而表現，*LLA-142* 則不會。利用反式核醣探針 (riboprobe) 進行原位雜合 (*in situ* hybridization) 的實驗證實 *LLA-89* 和 *LLA-142* mRNA 在花藥壁的絨氈層中呈現強烈的訊號。兩基因的 mRNA 也可在花藥發育中的小孢子偵測到。LLA-89 和 LLA-142 兩蛋白質的功能有進一步加以討論。

關鍵詞：花藥專一性基因；激勃素；鐵炮百合；小孢子；絨氈層。