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Research article

A pollen-specific polygalacturonase from lily is related to major grass pollen allergens

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Abstract

A pollen-specific gene from lily (*Lilium longiflorum* Thunb. cv. Snow Queen), designated *LLP-PG*, was characterized. Southern blots of lily genomic DNA indicated that *LLP-PG* is a member of a small gene family. A thorough sequence analysis revealed that the *LLP-PG* gene is interrupted by two introns and encodes a protein of 413 amino acids, with a calculated molecular mass of 44 kDa, and a p*I* of 8.1. Evaluation of the hydropathy profile showed that the protein has a hydrophobic segment at the N-terminus, indicating the presence of a putative signal peptide. A sequence similarity search showed a significant homology of the encoded protein to pollen polygalacturonases (PGs) from various plant species and to an important group (group 13) of grass pollen allergens. The *LLP-PG* transcript is pollen-specific and it accumulates only at the latest stage during pollen development, in the mature pollen. In contrast to other "late genes" *LLP-PG* transcript can neither be induced by abscisic acid (ABA) nor by dehydration. Immunoblot analyses of pollen protein extracts from lily, timothy grass and tobacco with IgG antibodies directed against LLP-PG and against the timothy grass pollen allergen, Phl p 13, indicated that lily LLP-PG shares surface-exposed epitopes with pollen PGs from monocotyledonous and dicotyledonous plants. Enzyme-linked immunosorbent assay (ELISA) analyses and inhibition ELISA assays with patients' IgE demonstrated a very low IgE reactivity of lily rLLP-PG and a lack of cross-reactivity between rLLP-PG and the timothy grass pollen allergen, rPhl p 13. These data demonstrated that despite the significant sequence homology and the conserved surface-exposed epitopes LLP-PG represents a low-allergenic member of pollen PGs.

Keywords: Allergen; Gene expression; IgE cross-reactivity; *Lilium longiflorum*; Pollen-specific; Polygalacturonase

1. Introduction

The haploid male gametophyte of flowering plants, the pollen grain, develops in a sporophytic organ, the anther. It was estimated that pollen contains approximately 20,000 to 24,000 different mRNAs, sequences of which only 2000 to 7000 are transcribed in the pollen [1,2]. The majority of the transcripts, present in the pollen, thus derive from the anther-tissue. Molecular studies identifying anther- and pollen-specific genes support the notion that the development of a functional male gametophyte requires a rather large pool of such genes. Pollen-specific genes from various species have been characterized and can generally be categorized based on the time of expression. Early genes are expressed before the first mitosis and late genes are post-mitotically expressed [3]. Most of the so far characterized pollen-specific genes belong to the "late" genes. Products from these genes may be involved in pollen maturation, germination or tube growth [4,5]. Among these late pollen-specific genes, several cDNAs showing sequence similarity to polygalacturonases (PGs) have been described. PGs

Abbreviations: ABA, abscisic acid; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin; MOPS, 3-[*N*-morpholino]propanesulfonic acid; PG, polygalacturonase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 5×SSC, 750 mM NaCl and 75 mM sodium citrate; pH 7.0, TBST; 50 mM Tris–HCl, pH 8.0; 150 mM NaCl and 0.05% Tween 20.

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are pectin degrading enzymes, which are widely distributed in plants, fungi, and bacteria. In plants, PGs play important roles in many stages of plant development that include organ abscission zones, pod and anther dehiscence, microspore/pollen development, pollen tube growth, and symbiosis with rhizobia [6–8]. In many cases the expression of PGs is influenced by environmental stresses such as heat, high levels of CO_2, and low temperature [9–11] and often abscisic acid (ABA), a stress-responsive hormone, has been shown to be involved in the signaling. This influence of environmental stresses on the expression pattern has not yet been investigated in the case of the sub-group of pollen-specific PGs.

Members belonging to the pollen-specific PGs have so far been described in maize, tobacco, *Brassica napus*, cotton and willow [12–16]. Recently, it has been shown that a newly identified major allergen from timothy grass pollen, Phl p 13, also belongs to the family of pollen-specific PGs [17,18]. Allergens displaying high sequence similarity and IgE cross-reactivity with Phl p 13 have meanwhile been described in all common grass species and have been designated according to the allergen nomenclature system as group 13 grass pollen allergens [19]. It has been shown that these group 13 allergens display no IgE cross-reactivity with pollen-specific PGs from dicotyledonous plants [20]. A potential IgE cross-reactivity with other monocotyledonous plants had not been studied yet.

In a recent publication we describe the identification of a new cDNA encoding a lily (*Lilium longiflorum*) PG [21]. In the present work, we now used molecular, biochemical and immunological analyses to characterize the lily *LLP-PG* gene and analyze its expression pattern. We found that the *LLP-PG* gene belongs to a small gene family, contains two introns and encodes a hydrophilic polypeptide that has a putative signal peptide. The transcript was only expressed in pollen and accumulated only at the stage of pollen maturation, which identified *LLP-PG* as a late pollen-specific gene. In contrast to PGs expressed in other plant tissues, transcription of *LLP-PG* can not be induced by ABA. We further showed that lily LLP-PG shares surface-exposed epitopes with pollen PGs from monocotyledonous and dicotyledonous plants. However, the rLLP-PG protein exhibits only very few, unique IgE epitopes and shares basically no IgE cross-reactivity with the grass pollen-specific group 13 allergens.

2. Results

2.1. Genomic organization of LLP-PG gene

Sequence analysis of the *LLP-PG* gene (accession number DQ166206) revealed that the gene contains two introns, the first intron starts 454 bp after the start codon and has a length of 113 bp and the second intron starts 1083 bp after the start codon and has a length of 105 bp (Fig. 1). The splice site of the second intron, with GT at the 5′ end and AG at the 3′ end of the intron, completely conformed to the splice site of PGs consensus sequence, whereas the first intron with GC at the 5′ end and AG at the 3′ end, conformed to the most common class of

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                GGATATAGGCCTCCATTCGATACGATTCTCTCTTGAAGAGGGAAAGGTG   49
ATGGCCTCGATCTCTAGCGCCCGGCTGCTACTTTTGTCGTCGGTGCTATTGATCGTTAAG  109
M  A  S  I  S  S  A  R  L  L  L  L  S  S  V  L  L  I  V  K    20
CTCACCGCAGGCCTTGTTCCCGAGACCAAGGTGAATGTCAAGAGTTTCGGGGCGGTTGGC  169
L  T  A  G↓ L  V  P  E  T  K  V  N  V  K  S  F  G  A  V  G    40
GACGGCAAGACCGACAGCACTCAGGCAATACTACGTGCATGGGATCAAGCTTGTAATGGA  229
D  G  K  T  D  S  T  Q  A  I  L  R  A  W  D  Q  A  C  N  G    60
TTTGGAAAACAAAGAGTTATAGTCCCTGAAGGGGTGTATCTAACGGGGCCAATGGCTTTT  289
F  G  K  Q  R  V  I  V  P  E  G  V  Y  L  T  G  P  M  A  F    80
AGGGGCCCCTGCAATGGCTTCATATCGATGCAGGTAAGGGGGGAGCTGAGAGCCTACGGC  349
R  G  P  C  N  G  F  I  S  M  Q  V  R  G  E  L  R  A  Y  G   100
GATGTGGGGAAGTACCCAAATGCCAAGTGGGTCTCATACGAGGACCTCAACGGTTTGTTG  409
D  V  G  K  Y  P  N  A  K  W  V  S  Y  E  D  L  N  G  L  L   120
GTCACCGGCGGCGGGAGGTTCAATGCTCAAGGCAGCCAAGCATGGACCCAAAATGACTGC  469
V  T  G  G  G  R  F  N  A  Q  G  S  Q  A  W  T  Q  N  D  C   140
TCTACGAAGAAGAACTGCGCACTGCTTACCACGgcaagtccatgaacgcatacaaccctt  529
S  T  K  K  N  C  A  L  L  T  T                              151
gtcatttttatgtttgtcaaaattgtcgtacttagttttcttgcttaatctaatttcgtc  589
tctctcatgtgtgccctcctccgtag TCCGTCAAGTTCGACCACTGCACCAACGCCACC  648
                           S  V  K  F  D  H  C  T  N  A  T     162
ATCCGCCGCATAAACTCCATCGACAGCAAGTTTTTCCACATCGCCATCGACCAATGCACA  708
I  R  R  I  N  S  I  D  S  K  F  F  H  I  A  I  D  Q  C  T   182
GACATCACCGTCCACCACATCAACATCACCGCCCCCGGCACCAGCCCCAACACCGACGGC  768
D  I  T  V  H  H  I  N  I  T  A  P  G  T  S  P  N  T  D  G   202
ATCCATATCGGACGATCCACCAACGTCAACATCAGCAATGCCATCATCGGCACTGGCGAC  828
I  H  I  G  R  S  T  N  V  N  I  S  N  A  I  I  G  T  G  D   222
GACTGTATCTCCCTCGGCCCGGGCAGCTCCCATATCACCATCTCAAAGGTCCAGTGCGGC  888
D  C  I  S  L  G  P  G  S  S  H  I  T  I  S  K  V  Q  C  G   242
CCCGGGCACGGGATCAGTGTGGGGAGCTTGGGACGGTACATGAATGAGGAGAACGTGTGG  948
P  G  H  G  I  S  V  G  S  L  G  R  Y  M  N  E  E  N  V  W   262
GATGTCAAAGTGAAAAACTGTACATTGACGGGAACAACGAATGGGGTGCGAATCAAGACG 1008
D  V  K  V  K  N  C  T  L  T  G  T  T  N  G  V  R  I  K  T   282
TGGAAGGGGTCATCGCCGAGCGAGGCGTCTCAGTTCATCTTCCAGGATATTGAGATGAGG 1068
W  K  G  S  S  P  S  E  A  S  Q  F  I  F  Q  D  I  E  M  R   302
GAAGTGCAGAATCCGATTATCATTGATCAAGAGTACTGCTCCTATGACTACTGTGCTAAC 1128
E  V  Q  N  P  I  I  I  D  Q  E  Y  C  S  Y  D  Y  C  A  N   322
AATgtgagtctctatctctgtcatatatatatatatatatatgcactcaagtaagcttga 1188
N                                                              323
tataggcccaactgtatatatattaacctaaatgccaactatttgcagCCCCCAGCCCCA 1248
                                                  P  P  A  P   327
TCTAAAGTGAAGCTGAGCGATATCCAGTTCATGAACATCAAGGGGACCTCAACTTCCAAG 1308
S  K  V  K  L  S  D  I  Q  F  M  N  I  K  G  T  S  T  S  K   347
GTTGCAATCAATCTGATATGCAGCTCAGCAGTGCCATGCGAAGGGATTCAGCTAAGCGAT 1368
V  A  I  N  L  I  C  S  S  A  V  P  C  E  G  I  Q  L  S  D   367
ATCAGCCTCAAGTACATTAGGGCTGGCAAACCGACGATGGCCAATTGTTCACACGTCAGC 1428
I  S  L  K  Y  I  R  A  G  K  P  T  M  A  N  C  S  H  V  S   387
GGCACCACTAGTGGACTTGTGAGCCCTCCGTCATGCATAAAAGGGGCTGATGTTTCTTTG 1488
G  T  T  S  G  L  V  S  P  P  S  C  I  K  G  A  D  V  S  L   407
TTTACAACACAGATGTTATGAGACTTTTTTATACCTTATCTTGTTGTTGAGTTATTTTAT 1548
F  T  T  Q  M  L  *                                           413
GTTTCTCTCCCTCTTAGA                                            1566
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Fig. 1. Nucleotide and deduced amino acid sequence of the *LLP-PG* gene. Bold letters in the nucleotide sequence indicate the start codon and the stop codon. Introns are indicated by lower case and putative *N*-glycosylation sites are underlined. A vertical arrow indicates the potential cleavage site of the putative signal peptide.

nonconsensus splice sites [22]. The *LLP-PG* gene encodes a LLP-PG polypeptide (accession number AAX45476.1) of 413 amino acids with a calculated molecular mass of 44 kDa and a pI of 8.1. Five putative *N*-glycosylation sites (N-X-S/T) are present at amino acid positions 160, 190, 212, 268 and 382. Assessment of the hydropathy profile [23] of the protein showed that the polypeptide is mainly hydrophilic with a strong hydrophobic region near the amino terminus, which may indicate the presence of a signal peptide. The proposed site of cleavage is between positions 24 and 25 of the amino acid sequence.

Southern blot analysis of *Nco*I and *Eco*RV digested genomic lily DNA under high-stringency conditions showed that the ^{32}P-labeled *LLP-PG* cDNA [21] hybridized in case of both digests to a few fragments (Fig. 2). These results suggested that the *LLP-PG* gene belongs to a small gene family.

2.2. Organ-specificity and developmental regulation of LLP-PG transcripts

To determine whether the lily *LLP-PG* transcript is pollen-specific, a blot of total RNA from vegetative organs (roots,

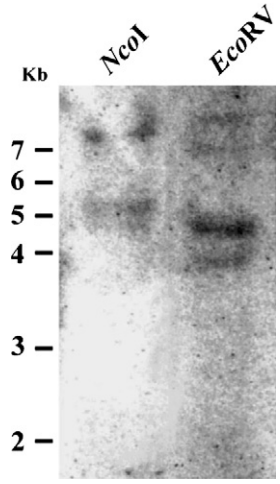

Fig. 2. Genomic Southern blot of lily DNA. *Nco*I and *Eco*RV digested lily leaf DNA (50 μg) was separated by agarose gel electrophoresis, blotted onto nylon and hybridized to the ^{32}P-labeled *LLP-PG* cDNA probe. Marker sizes in kb are given in the left margin.

stems and leaves) and floral organs (pollen, tepals, filaments and carpels comprising stigma, styles and ovaries) of mature flowers was hybridized to the ^{32}P-labeled *LLP-PG* cDNA (Fig. 3A). No hybridization signal was detected in RNA samples from other organs, indicating organ-specificity of the *LLP-PG* mRNA.

To determine the expression pattern of *LLP-PG* gene during anther development, a blot of total RNA isolated from anthers of different size classes of lily buds was hybridized to the ^{32}P-labeled *LLP-PG* cDNA. The *LLP-PG* transcript was first detected in the developing pollen of 135-mm buds and accumulated to a maximum level in the mature pollen of 165-mm buds (Fig. 3B). *LLP-PG* transcript was detected neither in the premeiotic phase nor in the phase of microspore development.

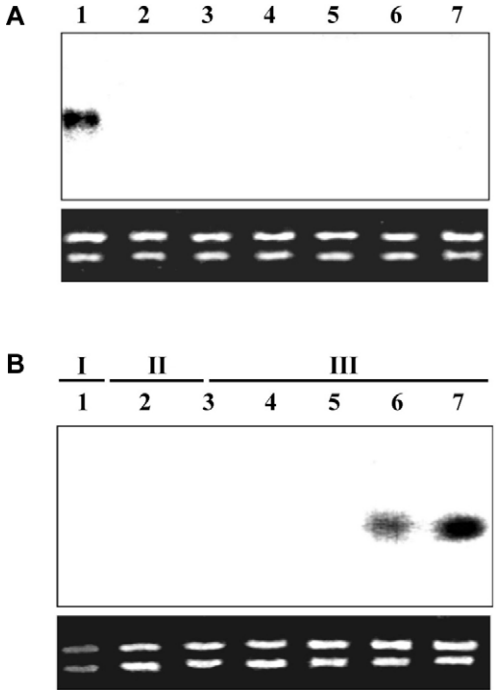

Fig. 3. Pollen-specific and temporal expression of the lily *LLP-PG* gene. Ten micrograms of total RNA (10 μg) from (A) pollen (lane 1), tepals (lane 2), carpels (lane 3), filaments (lane 4), roots (lane 5), stems (lane 6) and leaves (lane 7) and from (B) buds < 15-mm (lane 1), anthers of 30-mm buds (lane 2), anthers of 65-mm buds (lane 3), anthers of 90-mm buds (lane 4), anthers of 115-mm buds (lane 5), anthers of 135-mm buds (lane 6); and anthers of 165-mm buds (lane 7) were separated on formaldehyde-agarose gels, transferred to nylon membranes and hybridized to a ^{32}P-labeled *LLP-PG* cDNA probe. I indicates premeiotic anthers, II marks anthers at a stage of microspore development, when major cytological changes in the anther-wall layers take place. III indicates anthers at a stage of pollen maturation. Almost equal amounts of total RNA were loaded in each lane, as determined by ethidium bromide staining of the gel (bottom images). In case of buds < 15-mm (lane B1) only half the amount of total RNA was loaded.

2.3. *LLP-PG* transcript can not be induced by the addition of exogenous ABA in young anthers and pollen germination

In an earlier report, we demonstrated that the *LLP-PG* gene can not be induced by dehydration [21]. To investigate whether transcription of *LLP-PG* can be induced by ABA during pollen development or during in vitro pollen germination, Northern blot analyses were carried out. Total RNA was extracted from young anthers treated with 10 μM ABA or from mature lily pollen, or from pollen germinated for 24 h in the absence or presence of 10 and 100 μM ABA. The blots were hybridized to the ^{32}P-labeled *LLP-PG* cDNA and for control purposes to a ^{32}P-labeled *LLA23* probe. Hybridization with *LLA23*, a gene known to be inducible by ABA [24], revealed that the *LLA23* transcript was induced by ABA in young anthers whereas *LLP-PG* was not (Fig. 4A).

As to the analysis of in vitro pollen germination, the *LLA23* transcript was degraded after 24-h germination (Fig. 4B, lane 2), but the level of *LLA23* mRNA increased when 10 or 100 μM ABA was exogenously added to the germination buffer (Fig. 4B, lanes 3 and 4). The *LLP-PG* transcript, however, was not induced when ABA was applied, suggesting that, in contrast to the induction of *LLA23*, the *LLP-PG* gene can not be induced by ABA. It is interesting that substantial amount of *LLP-PG* mRNA remained in the pollen tubes after 24-h germination (Fig. 4B, lane 2). However, transcription of *LLP-PG* were inhibited with the addition of ABA (Fig. 4B, lanes 3 and 4).

2.4. Lily LLP-PG shares surface-exposed epitopes with pollen PGs from monocotyledonous and dicotyledonous plants

To investigate whether the sequence homology of LLP-PG with pollen PGs from various other plant species is also reflected in similar structural features and similar surface-exposed epitopes, the immunological relationship between lily LLP-PG and pollen PGs from monocotyledonous and dicotyledonous plants was studied (Fig. 5). For this, nitrocellulose-blotted pollen protein extracts from lily (Fig. 5A) and timothy grass (Fig. 5B), both monocotyledonous plants, as well as from tobacco (Fig. 5C), a dicotyledonous plant, were exposed either to a rat antiserum raised against rLLP-PG overexpressed in *E. coli* (lanes anti-rLLP-PG) or to a rabbit antiserum raised against the *E. coli* expressed timothy grass pollen PG, rPhl p

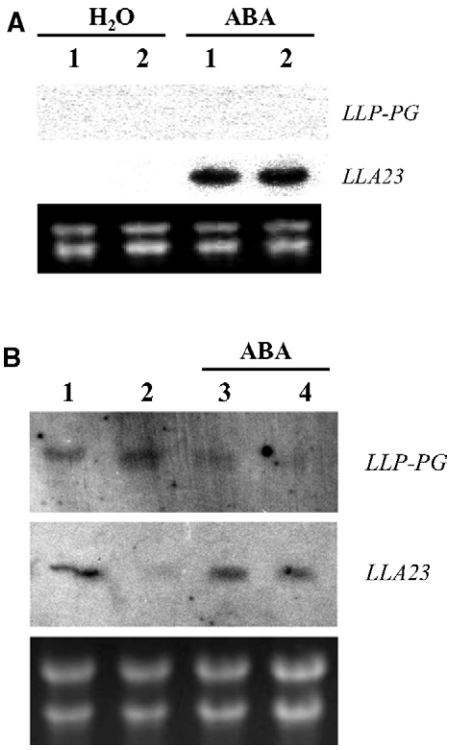

Fig. 4. Investigation of the effect of ABA on the accumulation of *LLP-PG* transcripts during pollen development (A) and during pollen germination (B) by RNA blot analysis. A, RNA was isolated from anthers of 50–60 mm buds (lanes 1) and 60–70 mm buds (lanes 2), which had been collected from water treated (H_2O) or ABA treated (ABA) lily plants. B, RNA was isolated from ungerminated pollen (lane 1) or from pollen germinated at 30 °C for 24 h in the absence of ABA (lane 2) or in the presence of 10 μM (lane 3) or 100 μM (lane 4) ABA. Five micrograms of total RNA were then separated on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized to a ^{32}P-labeled *LLP-PG* or *LLA23* cDNA probe. Almost equal amounts of total RNA were loaded in each lane, as determined by ethidium bromide staining of the agarose gels (bottom).

13 (lanes anti-rPhl p 13). The fact that anti-rPhl p 13 antiserum recognized not only the timothy grass pollen PG, but also a protein of 66 kDa in lily pollen (most likely lily LLP-PG) and a protein of 58 kDa in tobacco pollen (most likely tobacco PG), pointed to the presence of common antibody-binding epitopes and structural features in pollen PGs from monocotyledonous and dicotyledonous plants. The observation that anti-rLLP-PG antiserum only recognized PGs from lily pollen and timothy grass pollen, but not from tobacco pollen, suggested that the PGs from the two monocotyledonous plants were structurally closer related to each other than to the PG from a dicotyledonous plant (tobacco). The specificity of the observed antibody reactions was proven by the lack of positive signals in the immunoblot after hybridization with preimmune/nonimmune sera.

2.5. rLLP-PG exhibits unique IgE epitopes and shares no IgE cross-reactivity with rPhl p 13

The presence of common surface-exposed IgG binding epitopes on rLLP-PG and rPhl p 13, tempted us to investigate whether lily LLP-PG might also represent an allergenic molecule and might be recognized by patients displaying IgE reactivity to grass pollen PGs. For this, nitrocellulose-blotted rLLP-PG was incubated with sera from grass pollen allergic patients displaying IgE reactivity to the timothy grass pollen PG, Phl p 13. As can be seen in Fig. 6, IgE antibodies of some patients clearly recognized rLLP-PG (lanes a and d). However, IgE antibodies of the majority of patients (analysis with five representative sera was shown in Fig. 6) did not react with the protein. When a total protein extract prepared from mature lily pollen was blotted onto nitrocellulose and incubated with the same sera, lily LLP-PG was not detected by patients' IgE (data not shown). The observation that grass pollen patients' IgE antibodies apparently only recognized the overexpressed rLLP-PG, but not LLP-PG in a protein extract, indicated a rather low IgE binding activity of rLLP-PG.

For a quantitative comparison of patients' IgE reactivities to rLLP-PG and to rPhl p 13, enzyme-linked immunosorbent assay (ELISA) analyses were performed with sera displaying IgE reactivity to rLLP-PG. As indicated in Table 1, patients' sera exhibited much stronger IgE reactivity to rPhl p 13 than to rLLP-PG. It is interesting to note that patient 8, showing strongest IgE reactivity to rLLP-PG, did not show strongest IgE reactivity to rPhl p 13 and that patient number 7, showing strongest IgE reactivity to rPhl p 13, did not show strongest IgE reactivity to rLLP-PG (Table 1). These data suggested the presence of unique IgE epitopes on rPhl p 13, but also on lily rLLP-PG.

To investigate whether, besides unique IgE epitopes, lily rLLP-PG and timothy grass rPhl p 13 also share cross-reactive epitopes, ELISA inhibition experiments were performed with those sera that showed strongest IgE reactivity to rLLP-PG. Patients' sera 1, 3, 7, 8 and 9 were preincubated either with buffer, with rLLP-PG, with rPhl p 13 or with the unrelated control protein bovine serum albumin (BSA) and afterwards they were exposed to ELISA plate bound rLLP-PG or rPhl p 13. The results showed that preincubation with rLLP-PG was able to significantly reduce IgE binding to rLLP-PG (Table 2) and preincubation with rPhl p 13 nicely reduced IgE binding to rPhl p 13 (Table 3). However, preincubation with rLLP-PG hardly reduced IgE binding to rPhl p 13 and vice versa. In case of patients 1 and 3, even preincubation with the control protein BSA resulted in comparable or even higher degrees of inhibition. These data clearly indicated that rLLP-PG exhibited basically no IgE cross-reactivity to rPhl p 13. Patients reacting with rLLP-PG thus recognize IgE binding epitopes on this protein, which are different from IgE binding epitopes present on rPhl p 13 of timothy grass.

3. Discussion

Molecular, biochemical and immunological methods were used to characterize the lily PG gene, *LLP-PG*, and study its expression profile and its translation product. Southern blot analysis showed that, like other *PG*s [25], *LLP-PG* also belongs to a small gene family. PG genes are categorized into

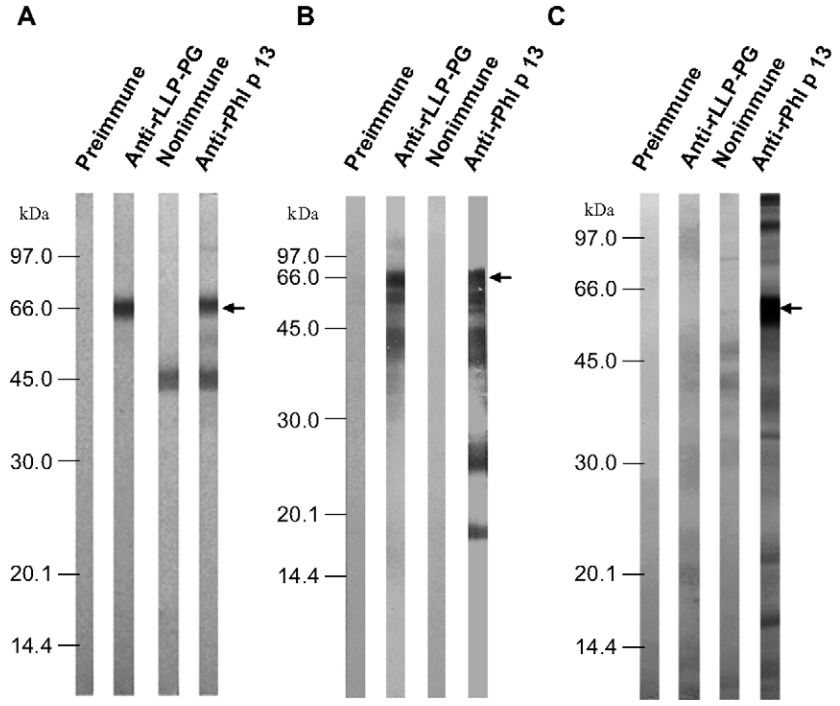

Fig. 5. Immunological relationship between LLP-PG and Phl p 13 proteins. Fifty micrograms of nitrocellulose-blotted protein extracts from mature pollen of lily (A), timothy grass (B) and tobacco (C) were exposed to rat anti-rLLP-PG and to rabbit anti-rPhl p 13 antisera and for control purposes, to rat preimmune and rabbit nonimmune sera. Arrows indicate the LLP-PG or LLP-PG-like proteins. Molecular weights are indicated in the left margins.

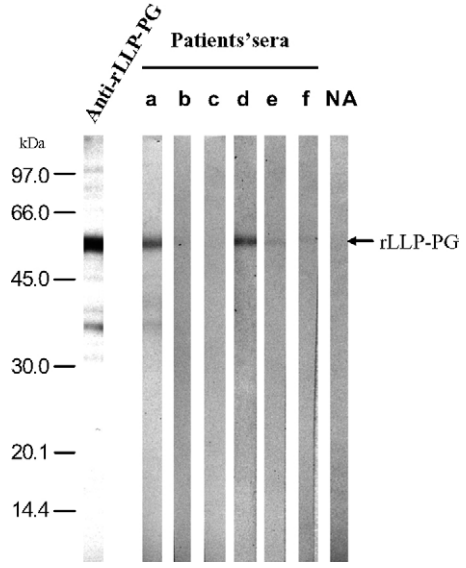

Fig. 6. IgE immunoblotting of purified rLLP-PG. Nitrocellulose-blotted rLLP-PG was either exposed to sera from grass pollen allergic patients (a–f) and to the serum of a non-allergic individual (NA) or to rat anti-rLLP-PG antiserum. Molecular weights are indicated in the left margin.

five classes [26] and the *LLP-PG* gene belongs to clade C that is composed entirely of genes expressed in pollen. In *Arabidopsis*, the *PG* members of clade C are characterized by the presence of three conserved introns [26]; two of them are also well conserved in *LLP-PG* in terms of relative position of the introns within the coding sequence. We identified *LLP-PG* as a pollen-specific gene (Fig. 3A) and saw that *LLP-PG* mRNA accumulates only at late stages of pollen maturation (Fig. 3B). Based on sequence analysis, a molecular mass of 44 kDa was predicted for the polypeptide encoded by *LLP-PG*. The fact that the mature, natural LLP-PG protein present in pollen has a molecular mass of 66 kDa points to the presence of post-translational modifications. The five putative *N*-glycosylation sites (N-X-S/T) found in the LLP-PG sequence indicate that LLP-PG might be glycosylated (Fig. 1).

Plant PGs are expressed in many plant tissues and they play important roles in many stages of plant development. The expression of some vegetative tissue-specific PGs has been shown to be inducible by environmental stress factors such as heat, high CO_2, and low temperature [9–11]. It is well known that most of these stress factors regulate gene expression via the ABA signal transduction pathway. During the last stages of maturation the pollen is exposed to an additional environmental stress, which also acts via the ABA signal transduction pathway, namely to desiccation, and late pollen-specific genes inducible by dehydration have recently been identified and characterized [24,27,28]. In a previous publication we found that *LLP-PG* can not be induced by dehydration [21]. ABA is a plant hormone known to induce transcription of PGs in vegetative tissues. We now investigated whether the lily PG gene might be inducible by any other stress, which regulates gene expression via the ABA pathway. However, whereas transcription of *LLA23*, a water-deficit/ripening-induced gene [24] used as a positive control, was inducible by the stress-responsive hormone ABA, transcription of *LLP-PG* was not (Fig. 4). We therefore concluded that *LLP-PG* and its related pollen-specific PG genes represent late pollen-specific genes, which are regulated by not yet identified factors independent of the well char-

Table 1
ELISA analyses of IgE binding to rPhl p 13 and rLLP-PG, respectively, with sera from eight Phl p 13-reactive patients

	Patients' sera								
Antigen	Buffer	1	2	3	4	7	8	9	10
rPhl p 13	0.074[a] ± 0.004	1.196 ± 0.054	2.977 ± 0.006	2.842 ± 0.223	1.379 ± 0.105	> 3.000	2.604 ± 0.209	2.777 ± 0.315	0.863 ± 0.122
rLLP-PG	0.051 ± 0.008	0.446 ± 0.071	0.161 ± 0.007	0.372 ± 0.001	0.205 ± 0.016	0.387 ± 0.061	0.763 ± 0.009	0.428 ± 0.197	0.488 ± 0.013

[a] OD values, corresponding to the amount of bound IgE, are displayed.

Table 2
IgE binding to rLLP-PG and percentage inhibition of IgE binding to rLLP-PG after preincubation of the sera with rPhl p 13, rLLP-PG and the unrelated control protein BSA

		% Inhibition of IgE binding after preincubation with		
Patients	IgE reactivity to rLLP-PG[a]	rPhl p 13	rLLP-PG	BSA
1	0.150 ± 0.005	11.33	71.33	26.00
3	0.049 ± 0.001	2.04	30.61	12.24
7	0.102 ± 0.004	3.92	46.07	0
8	0.262 ± 0.009	25.95	71.37	4.58
9	0.119 ± 0.009	17.64	49.57	1.68

[a] OD values, corresponding to the amount of bound IgE after preincubation with buffer, are displayed.

Table 3
IgE binding to rPhl p 13 and percentage inhibition of IgE binding to rPhl p 13 after preincubation of the sera with rPhl p 13, rLLP-PG and the unrelated control protein BSA

		% Inhibition of IgE binding after preincubation with		
Patients	IgE reactivity to rPhl p 13[a]	rPhl p 13	rLLP-PG	BSA
1	0.488 ± 0.005	50.61	35.86	30.32
3	0.202 ± 0.011	67.32	8.41	16.33
7	> 3.000	89.26	0	0
8	1.023 ± 0.051	46.13	5.18	0
9	1.597 ± 0.112	92.23	2.31	0.81

[a] OD values, corresponding to the amount of bound IgE after preincubation with buffer, are displayed.

acterized ABA signal transduction pathway. Beside the pathway of *LLP-PG* gene regulation, also the biological function of LLP-PG protein and its homologous pollen-specific counterparts in other plants still remains to be elucidated. PGs digest the 1,4-α-D-galactosiduronic linkages in pectate and other galacturonans and may function during pollen development, germination or tube growth by pectin depolymerization. In the aspect of pollen tube growth, these enzymes might provide wall precursors for pollen tube growth. This is supported by the insistent accumulation of *LLP-PG* mRNA even after 24-h germination (Fig. 4B). Therefore, LLP-PG seems to be actively involved in the growth of pollen tubes. Alternatively, the functional role of LLP-PG in pollen tubes may be to degrade the walls of the stylar cells to allow penetration of the pollen tube into the female tissue.

Using immunological techniques, like immunoblot analysis and ELISA, we also investigated immunological features of LLP-PG and, based on these data, got information on the structural relationship to other pollen-specific PGs. We found that the sequence homology of LLP-PG with pollen-specific PGs from monocotyledonous and dicotyledonous plants is reflected in shared surface-exposed epitopes. This is shown by the observation that antibodies raised against the timothy grass pollen PG, Phl p 13, recognized not only LLP-PG from the monocotyledonous plant lily, but also a pollen PG from the dicotyledonous plant tobacco. The closer structural relationship between the PGs of the two monocotyledonous plants, lily and timothy grass, is proven but the fact that anti-rLLP-PG antibodies recognized PGs from these two plants, but not from the dicotyledonous plant tobacco.

The fact that Phl p 13, the pollen-specific PG from timothy grass, represents an important allergen, tempted us to investigate whether LLP-PG might also be an allergenic molecule. We indeed found that rLLP-PG was recognized by IgE antibodies from a few patients. However, the IgE binding capacity of rLLP-PG appeared to be very low and results from ELISA inhibition experiments showed that the rLLP-PG IgE binding epitopes were different from those present on rPhl p 13 (Tables 2,3). In a recent study it has been shown that PGs from all common grass species share cross-reactive IgE epitopes, which do not occur on PGs from dicotyledonous plants [20]. We now provide important additional information by showing that other monocotyledonous plants, like lily, also lack these IgE binding epitopes, which thus appear to be indeed restricted to grasses. Due to the high sequence homology between LLP-PG and the group 13, PG grass pollen allergens, it should in future be challenging to identify and study based on sequence comparisons structural requirements of IgE binding epitopes.

4. Materials and methods

4.1. Plant materials

Roots, stems and leaves as well as tepals and carpels were collected from lily plants (*L. longiflorum* Thunb. cv. Snow Queen) grown in the field and immediately frozen in liquid nitrogen. For collection of lily anthers, flower buds of 10–170 mm containing anthers of 7–23 mm were harvested. Anthers from buds of more than 25 mm were then separated from filaments, whereas anthers from smaller buds were frozen together with the filaments. Buds of 20–25 mm contained pollen mother cells at the stage of meiosis, in buds of around 65–70 mm microspore mitosis was completed and the pollen entered the maturation phase of development. Pollen was collected 1 or 2 d after anthesis from 160- to 170-mm buds. All the plant material was stored at –80 °C until use.

4.2. DNA sequencing

The recombinant plasmid DNA of pGEM-T Easy Vector in which the *LLP-PG* cDNA resided was isolated using a Plasmid Miniprep Purification kit (GeneMark Technol. Co. Ltd., Taipei, Taiwan). The complete DNA sequence was determined from both strands of the cloned inserts with an ABI 3730XL DNA analyzer (Foster City, CA, USA) by Mission Biotech Co. Ltd. (Taipei City, Taiwan). Sequence comparison was carried out using the Cluster method of the DNA Star Program (DNASTAR Inc., Madison, USA) and the homology search was done with the BLAST program [29].

4.3. DNA blot analysis

Lily DNA was isolated from young leaves according to Maliga et al. [30]. The DNA was digested with restriction endonucleases, separated by 0.8% agarose gel electrophoresis and transferred onto nylon membranes (Micron Separation Inc.). Membranes were prehybridized for 4 h at 42 °C in 5× SSC, 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^{-1} of denatured salmon sperm DNA. The prehybridization solution was removed and replaced with hybridization buffer (5×SSC, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 20 mM sodium phosphate, pH 6.5, 0.1% (w/v) SDS, 50% formamide). Denatured salmon sperm DNA (100 μg ml^{-1}) and random-primed ^{32}P-labeled *LLP-PG* or *LLA23* probe (1.0×10^9 cpm μg^{-1}) (labeled using the Rediprime™ II of Random Prime labeling System from Amersham Biosciences) were added. Hybridization was carried out at 42 °C overnight with constant agitation. Afterwards, the membranes were washed at 42 °C in 2×SSC, 0.1% (w/v) SDS twice for 20 min, and at 55 °C in 0.1×SSC, 0.1% (w/v) SDS twice for 20 min. Membranes were then exposed to X-ray films (Konica AX) with 1 or 2 intensifying screens (DuPont).

4.4. RNA blot analysis

Total RNA was extracted from mature and germinating pollen, from developing anthers, from other floral organs and from vegetative tissues with the Ultraspec RNA isolation system (Biotecx Laboratories Inc.). Only tepal RNA was isolated using the LiCl extraction method described by Shirzadegan et al. [31]. RNA samples were electrophoresed in 1.0% formaldehyde-3-[*N*-morpholino]propanesulfonic acid (MOPS) gels using standard procedures [32] and transferred onto nylon membranes (Micron Separation Inc.). Prehybridization and hybridizations were performed as described above for the DNA blots. After hybridization, membranes were washed at 42 °C in 2×SSC, 0.1% (w/v) SDS twice for 20 min, and at 60 °C in 0.1×SSC, 0.1% (w/v) SDS twice for 20 min.

4.5. Protein extracts, recombinant proteins and immunoblot analysis

The phenol extraction method was used to extract total protein from mature pollen of lily and tobacco plants [33]. Total protein was extracted from pollen of timothy grass by shaking of the pollen in PBS containing 2 mM PMSF overnight at 4 °C. Insoluble particles were removed by centrifugation at 17,000×*g* for 30 min at 4 °C and the supernatant was stored at –20 °C until use. The protein concentration of the extracts was determined using a Lowry assay (Bio-Rad DC Protein Assay; Bio-Rad, Hercules, CA, USA) according to the supplier's directions. Recombinant lily rLLP-PG and timothy grass pollen rPhl p 13 were expressed in *E. coli* and purified to homogeneity as previously described [20,21].

For immunoblot analysis, 50 μg of pollen protein extracts or 1 μg of the recombinant proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [34] and blotted onto Immobilon-NC membranes (0.45 μm, Millipore, Billerica, MA, USA). Membranes were then either incubated with a 1:4000 diluted rat anti-rLLP-PG antiserum [21], a 1:1000 diluted rabbit anti-rPhl p 13 antiserum [20] or with 1:10 diluted sera from grass pollen allergic patients. For detection of bound rat IgGs, membranes were then incubated with a 1:6000 diluted alkaline-phosphatase (AP)-coupled anti-rat antibody (Chemicon International Inc., Temecula, CA, USA). For detection of bound rabbit IgGs, membranes were exposed to an AP-coupled anti-rabbit antibody with a dilution of 1:6000 (Promega, Madison, WI, USA). For detection of human IgE, membranes were incubated with a 1:1000 diluted AP-coupled anti-human IgE antibody (Sigma, St. Louis, MO, USA). Antibody binding was finally visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates.

4.6. ABA treatments

To investigate the influence of ABA on gene expression during pollen development, freshly cut lily plants with buds of 50–60 and 60–70 mm were dipped in water (control) or in aqueous solutions of 10 μM ABA for 24 h. Afterwards, anthers were collected from lily buds, immediately frozen in liquid nitrogen and stored at –80 °C.

The effects of ABA during pollen germination were studied in in vitro germination tests. Therefore approximately 200 mg pollen, collected 1 d after anthesis, was incubated in 5 ml of germination medium [0.29 M pentaerythritol, 300 μg ml^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 10 μg ml^{-1} H_3BO_3 and 100 μg ml^{-1} KNO_3] [35] at 30 °C with shaking for 24 h. For treatments with ABA, 10 or 100 μM ABA were added to the germination medium 2 h after the transfer of the pollen into the germination medium and incubations were also carried out at 30 °C for 24 h. Finally the pollen was washed twice with germination medium, centrifuged and frozen in liquid nitrogen.

4.7. ELISA and ELISA inhibition analyses

The IgE reactivity to rLLP-PG and rPhl p 13 was determined in sera from grass pollen allergic patients, and in a control serum from a nonatopic individual by ELISA. ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with rLLP-PG or rPhl p 13 (4 μg ml^{-1} in 0.1 M sodium bicarbonate, pH 9.6) and blocked with 1% BSA in TBST (TBS containing 0.05% (v/v) Tween 20). Plates were then incubated with sera (1:5 diluted in TBST, 0.5% BSA) and bound IgE antibodies were detected in duplicate with a HRP-coupled goat anti-human IgE antibody (1:2500 diluted in TBST, 0.5% BSA; KPL, Gaithersburg, MD) as described by Swoboda et al. [36].

For ELISA inhibition experiments, ELISA plates were incubated with sera (1:5 diluted in TBST, 0.5% BSA) that had been preadsorbed with 10 μg ml^{-1} of rPhl p 13, rLLP-PG, the unrelated control protein BSA or with buffer (TBST, 0.5% BSA). Bound IgE antibodies were detected with a HRP-coupled goat anti-human IgE antibody as described above. The reduction of IgE binding to rLLP-PG or rPhl p 13 after preincubation with the different inhibitors was calculated as $100 - (OD_{inh}/OD_B) \times 100$, where OD_{inh} and OD_B represent the extinction coefficients after preincubation with the inhibitors and with buffer, respectively.

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