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Short communication

Characterization of a lily tapetal transcript that shares sequence similarity with a class of intracellular pathogenesis-related (IPR) proteins

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Abstract

This study addresses isolating and characterizing a cDNA clone corresponding to a tapetum-specific transcript, designated as PR-10a from *Lilium longiflorum*. Anther ontogeny is histologically divided into three consecutive phases. The first encompasses early proliferative stages and differentiation of the locules. The second concerns microspore development from the onset of meiosis through microspore maturation. The final phase involves pollen maturation, originating with microspore mitosis through pollen formation. The lily PR-10a transcript is anther-specific and temporally expressed only at the phase of microspore development during which the tapetal cells become polarized, highly secretory, and exhibit loss of cell walls. The maximal level of PR-10a transcript coincides strictly with the peak of tapetal secretory function. Comparing mRNA and cDNA insert sizes reveals that PR-10a is close to full-length. Sequence analysis demonstrates similarity between the predicted lily PR-10a and asparagus AoPR1 protein, potato pSTH2 and pSTH21 proteins, parsley PcPR1 and PcPR3 proteins, bean PvPR1 and PvPR2 proteins, lupin LlR18B protein, pea I49 protein and a family of major allergens including Cor a 1 of hazel, Car b 1 of hornbeam, Aln g 1 of alder, Bet v 1 of birch and Api g 1 of celery. To the authors' knowledge, this is the first reported organ/tissue-specific IPR protein.

Pathogenesis-related (PR) proteins are a class of plant defense proteins that are induced in many plants exposed to fungal and bacterial pathogens, to certain chemical inducers or upon mechanical wounding [24, 29]. PR proteins fall into five major groups, two of which have been assigned to exhibit β-1,3-glucanase [14] and chitinase activity [17]. In recent years, a new class of small intracellular defense-related proteins, unlike any previously characterized PR protein, has been diagnosed in various dicot plants [27, 32] and also found in a monocot, asparagus [36]. All members of this ubiquitous protein class share a similar molecular mass (about 16 kDa), a similar isoelectric point, and similar sequence patterns, clearly indicating a common ancestry. Although the function of these intracellular pathogenesis-related (IPR) proteins is generally unknown, a ginseng ribonuclease with a high sequence identity with two IPR proteins from parsley has been identified [21]. Recently, Warner *et al.* [35] has localized an IPR protein gene (AoPR1) both in vegetative and floral organs in which the expression of the AoPR1 gene closely correlates with sites of phenylpropanoid biosynthesis.

In addition to those induced by pathogens or other stresses, certain subsets of PR proteins are frequently subject to developmental control in healthy plants, particularly in the floral organs. For examples, chitinase RNA transcripts exist in the nucleic acid extracts of whole flowers [19]. Chitinase, β-1,3-glucanase, and osmotin accumulate in the anthers and other parts of floral organs during development and senescence [18, 22]. The developmental regulation of IPR proteins has also been described in the floral and vegetative

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U76544 (lily PR-10a cDNA).

organs of pea [3], birch [7], soybean [10] and transgenic tobacco [35]. However, the reason for the developmental expression of these stress-related genes is unclear.

Anther is a highly specialized organ in which the sporogenous cells undergo meiosis to form tetrads of haploid microspores that, in turn, divide mitotically and then differentiate into pollen grains. The process of microsporogenesis is not solely dependent on the developing gametophyte. Many genes in the surrounding anther-wall layers are uniquely expressed, particularly in the tapetum [9, 15, 23, 26, 28] such that they provide an indispensable and fundamental contribution to the developing microspores. Previous reports [2, 34] have immunologically described a developmentally regulated tapetal protein in lily anther. This study reports on the cloning and characterization of the tapetal protein. We first cloned PR-10a cDNA and the complete nucleotide sequence was determined.

Poly(A)$^+$ RNA was isolated from 3.5-mm lily (*Lilium longiflorum* Thunb. cv. Snow Queen) anthers, and its complementary DNA synthesized and cloned into the *Eco*RI site of λZapII. The resulting library was screened with the anti-LLA-15 (lily PR-10) antiserum (1: 6000 dilution). After rescreening at low densities, seven plaques remained positive. The size of the cDNA insert in each of the seven clones was around 600 bp. One of these clones was taken for further analysis and sequencing.

Figure 1 displays the complete nucleotide sequence of lily PR-10a. The complete clone is 622 bp long, excluding the poly(A) tail. An open reading frame of 474 bp begins with an ATG initiation codon at nucleotide 29 and ends with a stop codon TAA at nucleotide 502. The ATG is preceded by a consensus Kozak sequence (CCACC) that is repeated three times [8, 12]. The poly(A) tail is located 120 nucleotides downstream from the TAA translation termination codon. In the 3′ non-coding region, no A/GATAAA consensus polyadenylation signal sequence can be found in the vicinity of the poly(A) addition site [13, 37]. Comparing mRNA (Figure 2A) and cDNA insert sizes indicates that the lily PR-10a is close to full-length. The deduced amino acid sequence of the PR-10a cDNA consists of 157 amino acids with a calculated molecular mass of 16.6 kDa and a pI of 5.2. The predicted amino acid sequence is rich in alanine (12%), serine (11%), glutamic acid (10%), and valine (10%) and has an overall excess of five acidic residues in relation to basic amino acids (20 acidic and 15 basic amino acids). The acidic nature and low molecular weight

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1                                                CGAGGAAGCCCAAGCAACCACCACCACC   28

29   ATGGCTTTCTCACTAGAGATTGAGTCTTCCGTCTCCGCCTCCAGGATGTTCAAGGCTGCC   88
1    M  P  F  S  L  E  I  E  S  S  V  S  A  S  R  M  F  K  A  A    20

89   CTCATCGAGTGGTACAACCTCGGTCCCAAGCTCGCCCCCGAGATCTTGCTCAGCGGTTCC  148
21   L  I  E  W  Y  N  L  G  P  K  L  A  P  E  I  L  L  S  G  S    40

149  ATTGTCGAAGGTGAGAGCGGGGCTGTCGGTGGGATCAGGCAACTCAATTTCTCCTCAGTG  208
41   I  V  E  G  E  S  G  A  V  G  G  I  R  Q  L  N  F  S  S  V    60

209  ATGCCATTCAGCTACGTGAAGGAGCGCTTGGACTTCATCGACCATGAGAAGTTTGAGTGC  268
61   M  P  F  S  Y  V  K  E  R  L  D  F  I  D  H  E  K  F  E  C    80

269  AAGGTGAGTGCAGTAGAAGGCGGCCATCTGGGAACCATTCTTGAATCTGCGTCTGCTCAC  328
81   K  V  S  A  V  E  G  G  H  L  G  T  I  L  E  S  A  S  A  H   100

329  TTCAAGATTGTGCCCAACAGCAGTGGAGGGTGTGTGGTGAAGGTGGTGACACAGTCGAAG  388
101  F  K  I  V  P  N  S  S  G  C  C  V  V  K  V  V  T  E  S  K   120

389  CTGAAACCGGGAGCTGTTCCCGCAGATGATGAAGCAAAGGCCAAGGAGGCGATGGTGATG  448
121  L  K  P  G  A  V  P  G  D  D  E  A  K  A  K  E  A  M  V  M   140

449  CTCTTCAAGGCTGCCGAAGCCTACCTCGTTGCCAACCCTGATGCCTATGCTTAATTCACC  508
141  L  F  K  A  A  E  A  Y  L  V  A  N  P  D  A  Y  A  *         157

509  TGCTGTGCTATCTATATGAATAAGTATGTTGGGATAGATGTTATGCGGTGAGTTTTGTAA  568

569  TTCGATCGCATGTAATGGATTGCATACTCTGTTTTCAGAGTAGACAAATAATCG        622
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Figure 1. Nucleotide sequence and deduced amino acid sequence of lily PR-10a cDNA. The double lines indicate the putative Kozak consensus sequence for initiation of translation. Bold letters in the nucleotide sequence indicate start codon and stop codon. Underlines indicate the putative glycosylation sites.

of this protein are in good agreement with a group of polypeptides previously determined by two dimensional gel analysis [33, 34]. Two putative N-glycosylation sites (N-X-S/T) are present at amino acid positions 56 and 106. Analyzing the predicted amino acid sequence reveals that no N-terminal signal peptide [31] can be discerned in PR-10a, indicative of a cytoplasmic protein. The intracellular feature of PR-10a is supported by ultrastructural localization of the protein as identified by anti-rabbit immunoglobulin G conjugated with gold particles [2].

In fact, the nucleotide sequence of lily PR-10a exhibits two open reading frames. The first ATG triplet occurs at nucleotide 29 and the second at nucleotide 74 (Figure 1). Because the former is preceded by a consensus Kozak sequence (CCACC) [8, 12] and because eucaryotic transcription generally favors the first initiation codon, it appears likely that translation is originates from that triplet. However, the possibility that the second ATG triplet initiates translation can not be excluded.

To determine whether the lily PR-10a transcript is anther-specific, a blot of total RNA from various

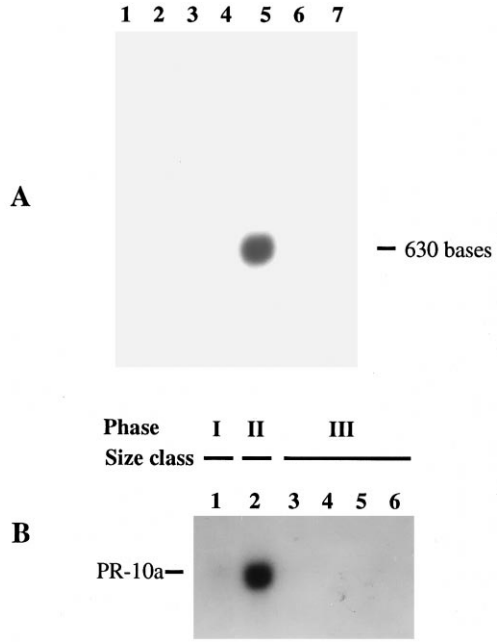


Figure 2. Anther-specific and temporal expression of lily PR-10a. Equal amounts of total RNA (15 μg) isolated (A) from various vegetative organs: roots (lane 1), stems (lane 2) and leaves (lane 3) and floral organs: tepals (lane 4), anthers (lane 5), filaments (lane 6) and carpels (lane 7) of 35-mm buds (16-mm anthers), and (B) from different size classes of anthers: 1 = 8 mm (15-mm buds); 2 = 16 mm (35-mm buds); 3 = 20 mm (55-mm buds); 4 = 21 mm (65-mm buds); 5 = 22 mm (95-mm buds); 6 = 23 mm (125-mm buds) were used in northern analysis. The autoradiogram was exposed for 5 days at $-70°$C. Phase I corresponds to the premeiotic anthers, Phase II corresponds to a period of microspore development and major cytological changes in the anther-wall layers. Phase III corresponds to pollen maturation.

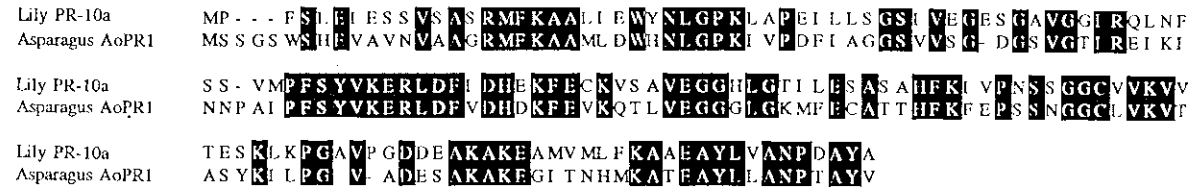
vegetative organs (roots, stems, and leaves) and floral organs (anthers, filaments, tepals, and carpels comprising stigmas, styles and ovaries) of 35-mm buds was hybridized with radiolabeled PR-10a cDNA. In the 35-mm lily buds, anthers were 16 mm in length and the level of PR-10a transcript was close to the maximum. A size of 630 bases of RNA species was detected in the anther RNA sample (Figure 2A, lane 5), a size similar to that of the full-length PR-10a cDNA. No hybridization signal was detected in RNA samples from other organs, indicating that PR-10a expression is anther-specific. Equal amounts of total RNA fractionated on the gel were judged by ethidium bromide staining.

It was previously observed that PR-10a protein in anthers accumulated only at the phase of microspore development at which meiosis begins in the pollen mother cells and major cytological changes occur in the tapetum [34]. To determine whether the level of lily PR-10a transcript follows the same developmental pattern, a blot of total RNA isolated from anthers of different size classes of lily buds was hybridized to the radiolabeled PR-10a cDNA (Figure 2B). The PR-10a transcripts were first detected at trace level in 8-mm anthers (15-mm buds) prior to the onset of meiosis (Figure 2B, lane 1), and accumulated to a maximal level in 16-mm anthers (35-mm buds) during which the tapetum of the anther wall layers became polarized and highly secretory (Figure 2B, lane 2). The level of PR-10a RNA in 16-mm anthers is about 60-fold of that in 8-mm anthers. No PR-10a transcript was detected either in 20-mm anthers (55-mm buds) or in maturing anthers (Figure 2B, lanes 3–6). The tapetal cells in 20-mm anthers (55-mm buds) exhibited loss of cell wall and began disintegrating (data not shown).

The expression of PR-10a transcripts strictly coincides with the peak of the tapetal secretory function. An increase in the level of lily PR-10a mRNA during development contributes to an increase in the protein level. As the expression of the tapetum-specific gene ceases, the accumulation of the protein significantly decreases. The decreasing protein level and the disintegration of the tapetal tissue are concurrent. The protein in the tapetal cells suggests a role in the sporopollenin pathway [2]. However, the function of the tapetal protein is still unknown.

The predicted amino acid sequence shown in Figure 1 was utilized to search protein databases. Sequence alignment was achieved using the Cluster method in DNA Star and the homology search was done with the BLAST program [1]. Sequences that revealed significant resemblance were a group of previously described IPR proteins: an asparagus AoPR1 protein [36] (54% identity); two potato PR proteins [19], pSTH2 (38% identity) and pSTH21 (36% identity); two parsley PR proteins [27], PcPR1 and PcPR3 (35% identity, respectively); two bean PR proteins [32], PvPR2 (32% identity) and PvPR1 (31% identity); lupin LlR18B protein [25] (31% identity) and pea I49 protein [11] (31% identity). Significant similarities also appeared with respect to a family of major allergens [4, 5, 6, 7, 16], including Cor a 1 of hazel (39% identity), Aln g 1 of alder (38% identity), Bet v 1 of birch (37% identity), Car b 1 of hornbeam (36% identity) and Api g 1 of celery (32% identity). Figure 3 shows the sequence alignments of lily PR-10a to the monocot asparagus AoPR1 protein and to the dicot PR proteins: potato pSTH2, Bet v 1, parsley PcPR1, bean PvPR2, lupin LlR18B, and pea I49.

A



B

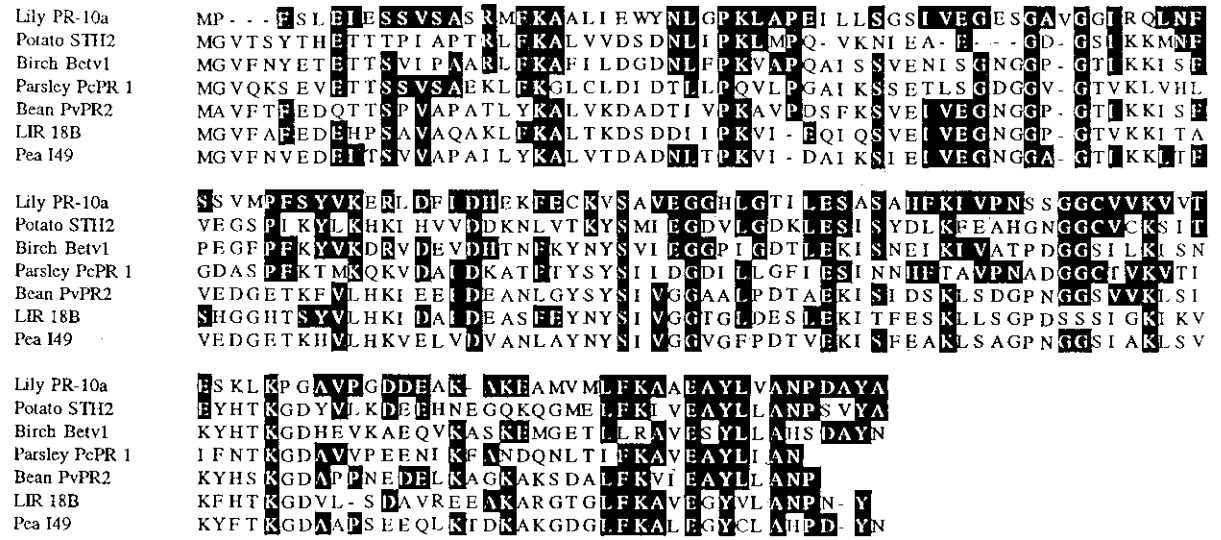


Figure 3. Alignment of amino acid sequence of lily PR-10a with related IPR proteins. The lily PR-10a amino acid sequence is compared with the predicted amino acid sequences of (A) AoPR1 [36], a monocot asparagus and of (B) the dicot PR proteins: potato STH2 [19], birch Bet v 1 [7], parsley PcPR1 [27], bean PvPR2 [32], lupin LlR18B [25], and pea I49 [11]. A dash in the sequence indicates a gap introduced in order to maintain a good alignment. Those amino acids that are identical to lily PR-10a are marked with black boxes.

These proteins are structurally unrelated to the tobacco PR-1 class [32]. Both lily and asparagus belong to the same monocot plant family, Liliaceae. It is no surprise that the predicted lily PR-10a protein exhibits a much higher sequence similarity to the asparagus AoPR1 protein than to other PR proteins (Figure 3). The differences in sequence similarity between lily PR-10a and AoPR1 and other PR proteins may reflect the divergence between monocots and dicots. Although the sequence similarities are not marked among IPR proteins, those between lily PR-10a and dicot IPR proteins are evident. Comparing lily PR-10a to asparagus AoPR1 and other IPR proteins reveals that twelve amino acid residues are strictly conserved among aligned sequences (Figure 3). Charged amino acids (6 residues) and glycine (3 residues) would appear to be important as they are conserved to a much greater extent than other residues.

Frequently, many stress-induced PR genes either by pathogens or by abiotic stresses are differentially expressed in normal development [3, 18, 20]. For instance, the AoPR1 gene is expressed in the mature pollen or in various other parts of floral or vegetative organs [35]. Similar to the AoPR1, other PR genes both in the floral and vegetative organs of healthy plants are also expressed [7, 20, 22]. In contrast, the lily PR-10a is unique in that the gene is organ/tissue-specific. The lily PR-10a transcript is expressed only in the tapetal cells during anther development, and not in mature pollen nor in any other parts of floral or vegetative organs. Because lily PR-10a protein is expressed in the developing anther of healthy plants, no information thus far has suggested that the protein is induced by pathogen

infection or other observable stresses. Thus, on the basis of van Loon's definition [30], the lily PR-10a may be classified as a PR-like protein.

This study has reported on a tapetum-specific transcript similar to a class of IPR proteins. To our knowledge, this is the first example of an organ/tissue-specific IPR protein. Future research will aim to utilize this cDNA clone to isolate the tapetal gene and learn more about the function of the lily PR-10a protein in the developing anther.

Acknowledgments

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References

1. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic local alignment search tool. J Mol Biol 215: 403–410 (1990).
2. Balsamo RA, Wang J-L, Eckard KJ, Wang C-S, Lord EM: Immunogold localization of a developmentally regulated, tapetal-specific, 15 kDa lily anther protein. Protoplasma 189: 17–25 (1995)
3. Barratt DHP, Clark JA: Proteins arising during the late stages of embryogenesis in *Pisum sativum* L. Planta 184: 14–23 (1991).
4. Breiteneder H, Ferreira F, Hoffmann-Sommergruber K, Ebner C, Breitenbach M, Rumpold H, Kraft D, Scheiner O: Four recombina nt isoforms of *Cor a* 1, the major allergen of hazel pollen, show different IgE-binding properties. Eur J Biochem 212: 355–362 (1993).
5. Breiteneder H, Ferreira F, Reikersdorfer A, Duchêne M, Valenta R, Hoffmann-Sommergruber K, Ebner C, Breitenbach M, Kraft D, Scheiner O: Complementary cloning and expression in *Escherichia coli* of *Aln g* 1, the major allergen in pollen of alder (*Alnus glutinosa*). J Allergy Clin Immunol 90: 909–917 (1992).
6. Breiteneder H, Hoffmann-Sommergruber K, O'riordain G, Susani M, Ahorn H, Ebner C, Kraft D, Scheiner O: Molecular characterization of Api g 1, the major allergen of celery (*Apium graveolens*), and its immunological and structural relationships to a group of 17-kDa tree pollen allergens. Eur J Biochem 233: 484–489 (1995).
7. Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M: The gene coding for the major birch pollen allergen *Bet v 1*, is highly homologous to a pea disease resistance response gene. EMBO J 8: 1935–1938 (1989).
8. Cavener DR, Ray SC: Eucaryotic start and stop translation sites. Nucl Acids Res 19: 3185–3192 (1987).
9. Crossley SJ, Greenland AJ, Dickinson HG: The characterization of tapetum-specific cDNAs isolated from a *Lilium henryi* L. meiocyte subtractive cDNA library. Planta 196: 523–529 (1995).
10. Crowell DN, John ME, Russell D, Amisino RM: Characterization of a stress-induced, developmentally regulated gene family from soybean. Plant Mol Biol 18: 459–466 (1992).
11. Fristensky B, Horovitz D, Hadwiger LA: cDNA sequences for pea disease resistance response genes. Plant Mol Biol 11: 713–715 (1988).
12. Joshi CP: An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucl Acids Res 15: 6643–6653 (1987).
13. Joshi CP: Putative polyadenylation signals in nuclear genes of higher plants: a compilation analysis. Nucl Acids Res 15: 9627–9640 (1987).
14. Kauffmann S, Legrand M, Geoffroy P, Fritig B: Biological function of pathogenesis-related proteins: four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO J 6: 3209–3212 (1987).
15. Koltunow AM, Truettner J, Cox KH, Wallroth M, Goldberg RB: Different temporal and spatial gene expression patterns occur during anther development. Plant Cell 2: 1201–1224 (1990).
16. Larsen JN, Stroman P, Ipsen H: PCR based cloning and sequencing of isogenes encoding the tree pollen major allergen *Car b* 1 from *Carpinus betulus*, hornbeam. Mol Immunol 29: 703–711 (1992).
17. Legrand M, Kauffmann S, Geoffroy P, Fritig B: Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA 84: 6750–6754 (1987).
18. Lotan T, Ori N, Fluhr R: Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell 1: 881–887 (1989).
19. Matton DP, Brisson N: Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. Mol Plant-Microbe Interact 2: 325–331 (1989).
20. Memelink J, Hoge JHC, Schilperoort RA: Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. EMBO J 6: 3579–3583 (1987).
21. Moiseyev GP, Beintema JJ, Fedoreyeva LI, Yakovlev GI: High sequence similarity between a ribonuclease from ginseng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. Planta 193: 470–472 (1994).
22. Neale AD, Wahleithner JA, Lund M, Bonnett HT, Kelly A, Meeks-Wagner DY, Peacock WJ, Dennis ES: Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell 2: 673–684 (1990).
23. Paul W, Hodge R, Smartt S, Draper J, Scott R: The isolation and characterization of the tapetum-specific *Arabidopsis thaliana* A9 gene. Plant Mol Biol 19: 611–622 (1992).
24. Rigden J, Coutts R: Pathogenesis-related proteins in plants. Trends Genet 4: 87–89 (1988).
25. Sikorski MM, Szlagowska AE, Legocki AB: cDNA sequences encoding for two homologs of *Lupinus luteus* (L.) IPR-like proteins. Plant Physiol 110: 335–335 (1995).
26. Smith AG, Gasser CS, Budelier KA, Fraley RT: Identification and characterization of stamen- and tapetum-specific genes from tomato. Mol Gen Genet 222: 9–16 (1990).
27. Somssich IE, Schmelzer E, Kawalleck P, Hahlbrock K: Gene structure and *in situ* transcript localization of pathogenesis-related protein 1 in parsley. Mol Gen Genet 213: 93–98 (1988).
28. Staiger D, Kappeler S, Muller M, Apel K: The proteins encoded by two tapetum-specific transcripts, Sa tap35 and Sa tap44,

from *Sinapis alba* L. are localized in the exine cell wall layer of developing microspores. Planta 192: 221–231 (1994).
29. Van Loon LC: Pathogenesis-related proteins. Plant Mol Biol 4: 111–116 (1985).
30. Van Loon LC, Pierpoint WS, Boller T, Conejero V: Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Rep 12: 245–264 (1994).
31. Von Heijne G: Signal sequences. The limits of variation. J Mol Biol 184: 99–105 (1985).
32. Walter MH, Liu J-W, Grand C, Lamb CJ, Hess D: Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen allergens. Mol Gen Genet 222: 353–360 (1990).
33. Wang C-S, Walling LL, Eckard KJ, Lord EM: Patterns of protein accumulation in developing anthers of *Lilium longiflorum* correlate with histological events. Am J Bot 79: 118–127 (1992).
34. Wang C-S, Walling LL, Eckard KJ, Lord EM: Immunological characterization of a tapetal protein in developing anthers of *Lilium longiflorum*. Plant Physiol 99: 822–829 (1992).
35. Warner SAJ, Gill A, Draper J: The developmental expression of the asparagus intracellular PR protein (AoPR1) gene correlates with sites of phenylpropanoid synthesis. Plant J 6: 31–43 (1994).
36. Warner SAJ, Scott R, Draper J: Characterization of a wound-induced transcript from the monocot asparagus that shares similarity with a class of intracellular pathogenesis-related (PR) proteins. Plant Mol Biol 19: 555–561 (1992).
37. Wickens M: How the messenger got its tail: addition of poly(A) in the nucleus. Trends Biochem Sci 15: 277–281 (1990).