

Cloning and expression pattern of Hor v 9, the group 9 pollen isoallergen from barley

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Abstract

In this study we report the cloning, sequence, and characterization of Hor v 9 allergen cDNAs from barley (*Hordeum vulgare*) pollen. Structural homologues of Kentucky bluegrass (*Poa pratensis*) group 9 pollen allergens were identified in a cDNA library of barley pollen expressed mRNAs. The Hor v 9 cDNA clone (hvp9742) contained an open reading frame encoding 313 amino acids which included a putative 27-residue signal peptide and one asparagine sequon for glycosylation. The mRNA corresponding to clone hvp9742 was produced abundantly in pollen during the late stages of anther development. The protein encoded by clone hvp9742 was synthesized as a fusion protein in the *E. coli* expression vector pMAL. Immunoblots using antibodies to this recombinant allergen, rHor v 9, showed that Hor v 9 protein accumulated during pollen development and was produced maximally at pollen maturity. Using these antibodies, we also provide evidence that Hor v 9 protein localized to the extracellular matrix of mature pollen. Southern blots suggested that Hor v 9 allergens exist as multiple isoforms in barley. Sequence comparisons showed that the Hor v 9 cDNA clones were also homologous to group 5 allergens of Timothy grass (*Phleum pratense*) pollen and canary grass (*Phalaris aquatica*) pollen, and the group 9 allergen of ryegrass (*Lolium perenne*) pollen.

Keywords: Allergy; cDNA sequence; hvp9742; Pollen development; Fusion protein

1. Introduction

We have cloned and characterized the Kentucky bluegrass pollen Poa p IX isoallergens (Mohapatra et al., 1990), now renamed Poa p 9 isoallergens (King et al., 1994). Structural homologues of Poa p 9 have been found in taxonomically related species including ryegrass (*Lolium perenne*) (Ong et al., 1993), Timothy grass (*Phleum pratense*) (Vrtala et al., 1993b) and canary grass

(*Phalaris aquatica*) (Suphioglu and Singh, 1995). Poa p 9 isoallergens are relatively small (28–35 kDa) basic proteins with a potential carboxyl-terminal transmembrane helix. The immunologically important antibody-binding epitopes of Poa p 9 isoallergen KBG60 have been mapped (Zhang et al., 1992). Using antibodies, considerable cross-reactivity of grass pollen proteins has been observed in taxonomically related Poaceae species (Zhang et al., 1991). We have recently observed cross-reactivity of group I, group IV, and group IX proteins, DNA and RNA in agriculturally important crops, especially barley (*Hordeum vulgare*) (Astwood et al., 1995). Serum from Kentucky bluegrass pollen allergic patients cross-reacted with proteins in these crops (Astwood et al., 1995). Structural homologues of grass pollen allergens have been found in other agricultural species such as the group I allergen identified in maize (*Zea mays*) (Broadwater et al., 1993) and referred to as Zea m 1. The existence of pollen allergen homologues in important agricultural crops represents a newly defined source of aeroallergens (McSharry, 1992; Stewart, 1993) which could be important in occupational allergy (Bousquet et al., 1985). In addition, recombinant

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Abbreviations: BSA, bovine serum albumin; cDNA(s), complementary DNA(s); CTAB, hexadecyltrimethylammonium bromide; EDTA, ethylenediamine tetraacetic acid; kb, kilobase; kDa, kilodalton; IgG, immunoglobulin fraction G; IPTG, isopropyl β-D-thiogalactopyranoside; MBP, maltose binding protein; mRNA, messenger RNA; ORF(s), open reading frame(s); PAGE, polyacrylamide gel electrophoresis; pI, Isoelectric point; Pipes, piperazine-*N,N'*-bis[2-ethanesulfonic acid]; RNase, ribonuclease; SDS, sodium dodecylsulfate; SSC, saline sodium citrate buffer; TBS, Tris-buffered saline; TCA, trichloroacetic acid; TE, Tris-EDTA buffer; TTBS, Tween and Tris-buffered saline; Tris, Tris(hydroxymethyl)aminomethane.

allergens represent a useful antigen source for grass pollen immunotherapy as a treatment for hayfever (Scheiner and Kraft, 1995; Van Ree et al., 1995).

Pollen allergens also represent an important class of genes in the biology of pollen. Over 75 pollen allergen DNA and protein sequences appear in GenBank, PIR or SwissProt (Astwood and Hill, 1996). Novel functions for several pollen allergens have been suggested on the basis of sequence similarity to known proteins: birch pollen allergen Bet v 1 is homologous to the disease resistance genes of pea (Breiteneder et al., 1989), Bet v 2 may encode a homologue of profilin, a regulator of actin polymerization (Valenta et al., 1991), and Bet v 3 appears to be a calmodulin-like calcium binding protein (Seiberler et al., 1994). Recent evidence suggested that the group V allergen of Timothy, Phl p 5b, is a pollen RNase (Bufe et al., 1995). We are interested in defining the role of group 9 allergens and their structural homologues in the biology of pollen. Barley was chosen as the experimental model since anther development was better defined for barley than for Kentucky bluegrass (Astwood and Hill, 1995). Proteins and mRNAs homologous to Poa p 9 allergens have been identified in barley pollen using allergic patient sera, Poa p 9 antibodies and Poa p 9 cDNA probes; and a 30-kDa barley pollen protein was defined as the pollen allergen Hor v 9 (*Hor v IX*) (Astwood et al., 1995).

In this study we report the cloning, sequence, and characterization of group 9 pollen allergen cDNAs from barley. Further, a recombinant Hor v 9 fusion protein was produced by an *E. coli* protein expression vector. Mice were immunized with this recombinant protein and the resulting serum was used to examine Hor v 9 protein accumulation in barley tissues.

2. Results and discussion

2.1. Sequence of a group 9 cDNA in barley

To identify cDNA homologues of Poa p 9 allergens in barley, a cDNA library from mature pollen was prepared and screened with a 558-bp DNA fragment (c7.2) of the Poa p 9 isoallergen KBG 41 (Mohapatra et al., 1990). Screening with c7.2 yielded 25 positively hybridizing plaques. Insert size and nucleotide sequencing showed that none of the initial 25 clones were long enough to contain a complete open reading frame (ORF). Clone hvp97 (Table 1) was selected to re-screen the library because it showed considerable nucleotide sequence identity with KBG41 (55% overall, including 85% in the potential ORF). The two cDNA clones that hybridized most intensely with clone hvp97 were clones hvp971 (841 bp) and hvp974 (894 bp) but also contained incomplete ORFs. To obtain a full-length cDNA, the mature barley pollen cDNA library was re-screened with

clone hvp974, yielding the full-length cDNA hvp9742 (1339 bp). Table 1 summarizes these data.

The nucleotide sequence of hvp9742 revealed an ORF with a start codon at nucleotide 103 and a stop codon at nucleotide 1041 (Fig. 1). The computer-predicted protein product of the ORF was 313 amino acid residues long with a molecular mass of 33.1 kDa and isoelectric point (pI) at pH 10.31. The first 27 residues encoded a putative signal peptide, having features common to most eukaryotic signal peptides (Von Heijne, 1985) including a carboxyl-terminal hydrophilic region, a short amino-terminal hydrophilic region, a central hydrophobic region, the helix-breaker proline at the –6 position, a small neutral residue at –7 (valine), and glycine and arginine residues at positions –3 and –5. The mature polypeptide was 286 residues long, yielding a predicted molecular mass of 30.1 kDa and pI at pH 9.96. By comparison, Poa p 9 isoallergens range from 28 to 32 kDa in size and from 9.6 to 10.2 in pI and also contain signal peptides. An asparagine glycosylation sequon (Hunt and Dayhoff, 1970) was found at position +20 of the ORF. The nucleotide sequence also contained a putative 3' polyadenylation signal (AATGAA) beginning at nucleotide position 1310.

Initial Northern blot analysis showed that clone hvp97 was abundantly expressed in pollen. No hybridizing bands were observed in RNA from leaves and pistils; whereas bands were readily apparent in RNA from mature pollen (Fig. 2). Clone hvp9742 was approximately the same size as the mRNA identified by the Northern blot (Table 1). Based on this evidence, we concluded that clone hvp9742 represented a complete cDNA for Hor v 9.

2.2. Sequence similarity of hvp9742 to Poa p 9 and other pollen allergens

The polypeptide encoded by barley pollen clone hvp9742 shared 67.5, 57.1 and 69.5% amino acid sequence identity with Poa p 9 isoallergens KBG31, KBG41 and KBG60, respectively, and was named Hor v 9. It had been shown previously that the group 5 isoallergen (Phl p 5) of Timothy grass shares 90% amino acid sequence identity (Vrtala et al., 1993b) with Poa p 9 isoallergen KBG41. Recently an alignment of seven group 5 and group 9 grass pollen allergens from Kentucky bluegrass, Timothy and ryegrass showed that overall similarity among these allergens ranges from 75 to 92% (Bufe et al., 1995). A similarity search using BLASTA (Altschul et al., 1990) of the genetic databases (GenBank rel 94, SwissProt rel 33 and PIR rel 47) revealed that the polypeptide encoded by clone hvp9742 shared substantial sequence identity with 10 group 5 and group 9 grass pollen allergens (Fig. 3). The amino acid sequence alignment of Hor v 9 (hvp9742) with Poa p 9 isoallergens (KBG 30, KBG41, and KBG60), Lol

Table 1
Characteristics of Hor v 9 clones selected from a barley pollen cDNA library

Clone no.	Insert (nt)	% similarity ^a of cDNAs to clone hvp9742			
		mRNA (nt)	Nucleotide	Amino acid	Accession no.
hvp9742	1339	1450			U57845
hvp974	894	1450	100 (445–1339)	100	U06640
hvp971	841	1450	100 (348–1189)	100	
hvp97	625	1450	100 (694–1214)	100	

^aPosition of overlap with hvp9742 indicated by parentheses.

p 9, Phl p 5, Phl p 5a, Phl p 5b and Pha a 5 isoallergens (clones 14, 28 and 29) demonstrated that the carboxyl-terminal residues (residue Ser₁₀₇ to Val₂₈₀ relative to Hor v 9) of all 11 proteins shared the greatest degree of sequence similarity (over 70% of the residues were similar and 48 residues were identical in all 11 sequences at equivalent positions), whereas the amino-terminal region was highly variable (no residues were identical in all 11 allergens at equivalent positions). The highly variable amino-terminal region may impart unique functions to individual members of the group 9 isoallergen gene family. For example, the boundary of the variable and conserved regions of these genes may represent an intron splice-site important in their evolution (Gilbert, 1985).

We have previously noted that the Poa p 9 isoallergen amino acid sequences predicted a carboxyl-terminal transmembrane helix (Silvanovich et al., 1991). Therefore, all pollen allergens found to have sequence similarity to hvp9742 were tested for the presence of a potential transmembrane helix according to the algorithms of the ANTHERO sequence analysis collection (Geourjon et al., 1991). All proteins contained a potential transmembrane motif as found in Pro₂₆₃ to Gly₂₈₂ in the ORF of hvp9742.

2.3. Southern analysis of barley pollen cDNAs

To determine the copy number of Hor v 9 in the barley genome, Southern blots were probed with clone hvp97 (comprising 3'-nucleotides of hvp9742). Barley genomic DNA digested with the restriction enzymes *Bam*HI and *Hind*III showed two hybridizing bands (7.8 and 4.2 kb, and 5.8 and 4.1 kb, respectively). These data (Fig. 4) suggested one or two copies of Hor v 9 exist in the barley genome. Additional blots using less stringent hybridization and final wash suggested that many homologous genes were present in the barley genome (data not shown). Multiple isoforms for group 9 allergens have been observed in Kentucky bluegrass (Silvanovich et al., 1991).

2.4. Synthesis of recombinant MBP::974 protein in *E. coli*

To express and purify the protein encoded by Hor v 9, the incomplete clone hvp974 (see Table 1 for sequence position relative to hvp9742) was subcloned into an *E. coli* expression vector (pMAL-c2, New England Biolabs) which was modified in the multiple cloning site. The modification of pMAL was designed so that the ORF of hvp974 would be in frame with the translation initiation site of maltose binding protein (MBP) encoded by pMAL. pMAL was cut with *Eco*RI, to give a linear plasmid. The 3'-(TTAA) cohesive ends of the *Eco*RI cleavage site were removed by mung bean nuclease according to Sambrook et al. (1989). The last base of the resulting blunt-ended vector DNA represented the first base of the codon reading frame of MBP. The plasmid pMAL was further digested with *Pst*I, to give a cohesive end suitable for directional cloning. Barley pollen clone hvp974 insert DNA was excised from pBluescript using *Eco*RV, which yielded a blunt end beginning at the second base of the codon reading frame of hvp974 protein, and also with *Pst*I to give an hvp974 insert with a sticky-end after the stop codon of the hvp974 ORF. The *Eco*RV/*Pst*I insert DNA of clone hvp974 was ligated into the modified pMAL vector. The DNA sequence of the 5'-joint region of the recombinant pMAL::974 construct was determined to ensure that the hvp974 ORF was in frame with the translation start codon of MBP. The pMAL::974 construct was transformed into *E. coli* (XL1-Blue, MRF' Stratagene) and analyzed for the expression of recombinant protein. Since clone hvp974 contained the carboxyl-terminal 219 residues (23 kDa) of the complete 313-amino acid hvp9742 ORF, and MBP alone is 42 kDa, an MBP::974 fusion was anticipated to be approximately 65 kDa.

Transformed *E. coli* containing the pMAL::974 construct were grown in LB with isopropyl- β -D-thiogalactopyranoside (IPTG) to induce plasmid-driven synthesis of the MBP::974 fusion protein. SDS-PAGE gels of protein extracts from these transformants showed a

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1          cggcatctgcccaccatttggaggagggaaggcaacgtaacc
44  tcggcgcatcgcgatagccaacacacattaacaccgttacagagaacaatagcacaagaa

103  ATG GCC AAC AGC GGC AGA GAA CAC AGC GCA GTT CCA CGT CGC CGT
-27  MET Ala Asn Ser Gly Arg Glu His Ser Ala Val Pro Arg Arg Arg

148  AAC CTT GTT GCT CTG GTG CCG CGC CAT GGT TGT TAT GCA GAG TTT
-12  Asn Leu Val Ala Leu Val Pro Arg His Gly Cys Tyr Ala Glu Phe

193  AGT CTG TAT GTA TGT GTT GGC AAC ATT AAT GCA CCG TTC CCT GTA
4    Ser Leu Tyr Val Cys Val Gly Asn Ile Asn Ala Pro Phe Pro Val

238  TTC AAC AGG ACA ACA TTT ATC GCA AAT GCA GGG ATA GAG GCC GAG
19   Phe Asn Arg Thr Thr Phe Ile Ala Asn Ala Gly Ile Glu Ala Glu

283  TTG GAA CCC CAT TTT CTT CTT CTT CTT TTT ACA TTT TCT TCT TCT
34   Leu Glu Pro His Phe Leu Leu Leu Leu Phe Thr Phe Ser Ser Ser

328  TCT TCA TTT TTT ACA TTA TTG AAG ACC ATG ATT CAC TTC ACG GAC
49   Ser Ser Phe Phe Thr Leu Leu Lys Thr Met Ile His Phe Thr Asp

373  CGA TCA GAT AAT AAA AAT AAA GCA ATG ATG AGG GGC CGC GAA TTC
64   Arg Ser Asp Asn Lys Asn Lys Ala Met Met Arg Gly Arg Glu Phe

418  CGC AAG GCT TTC GCG GAG GTC CTC AAG GGC GCC GCC ACC GGC CAG
79   Arg Lys Ala Phe Ala Glu Val Leu Lys Gly Ala Ala Thr Gly Gln

463  ATC GCC GGC CAG TCC AGC TCC ATG GCC AAA CTC TCC AGC AGC CTC
94   Ile Ala Gly Gln Ser Ser Ser Met Ala Lys Leu Ser Ser Ser Leu

508  GAA CTC TCC TAC AAG CTC GCC TAC GAC AAA GCC CAG GGC GCC ACC
109  Glu Leu Ser Tyr Lys Leu Ala Tyr Asp Lys Ala Gln Gly Ala Thr

553  CCC GAG GCC AAG TAC GAC GCC TAC GTC GCC ACC CTC ACC GAG TCG
124  Pro Glu Ala Lys Tyr Asp Ala Tyr Val Ala Thr Leu Thr Glu Ser

598  CTC CGC GTC ATC TCC GGC ACC CTC GAG GTC CAC TCC GTC AAG CCC
139  Leu Arg Val Ile Ser Gly Thr Leu Glu Val His Ser Val Lys Pro

643  GCC GCC GAG GAG GTT AAG GGC GTC CCC GCC GGC GAG CTG AAG GCC
154  Ala Ala Glu Glu Val Lys Gly Val Pro Ala Gly Glu Leu Lys Ala

688  ATT GAC CAG GTC GAC GCC GCC TTC AGG ACC GCC GCC ACC GCC GCT
169  Ile Asp Gln Val Asp Ala Ala Phe Arg Thr Ala Ala Thr Ala Ala

733  GAC GCT GCC CCG GCC AAC GAC AAG TTC ACC GTC TTC GAG TCG CTT
184  Asp Ala Ala Pro Ala Asn Asp Lys Phe Thr Val Phe Glu Ser Leu

778  CAA CAA GGT CCA TCA AGG AAA CCA CGG GGC GGC GCG TAC GAG AGT
199  Gln Gln Gly Pro Ser Arg Lys Pro Arg Gly Gly Ala Tyr Glu Ser

823  TAC AAG TTC ATC CCC GCC CTC GAG GCC GCC GTC AAG CAG GCC TAC
214  Tyr Lys Phe Ile Pro Ala Leu Glu Ala Ala Val Lys Gln Ala Tyr

868  GCC GCC ACG GTC GCC GCC GCG CCG GAG GTA AAA TTC ACC GTC TTT
229  Ala Ala Thr Val Ala Ala Ala Pro Glu Val Lys Phe Thr Val Phe

913  CAG ACC GCG CTG AGC AAG GCC ATC AAT GCC ATG ACT CAA GCC GGG
244  Gln Thr Ala Leu Ser Lys Ala Ile Asn Ala Met Thr Gln Ala Gly

958  AAG GTT GCC AAG CCC GCC GCC GCA GCC ACC GCC ACC GCC ACC GTT
259  Lys Val Ala Lys Pro Ala Ala Ala Ala Thr Ala Thr Ala Thr Val

1003  GCC GCG GGG GCC GCC GCC ACT GCC GGT AAC TAC AAA GTC TGA GCT
274  Ala Ala Gly Ala Ala Ala Thr Ala Gly Asn Tyr Lys Val *

1048  ccgccaacactacacttgacgagcacgcacatactatgctgtatgtaagtgggtggc
1107  ctccggcgggcgagcgatcttccttttggtaacatgagcttggatcaaaagtgttga
1166  ttacaatcttgccttaagaatcggttaagggaagggaatgtaattaagccaaaggca
1225  tataggccttgactcgctcgatgcttccttcttggtttgcgttccatgcagccgcgaa
1284  ccggccgcgtgttcatgcacctgtaacaatgaaatatttggtaacctcatgaaaaat

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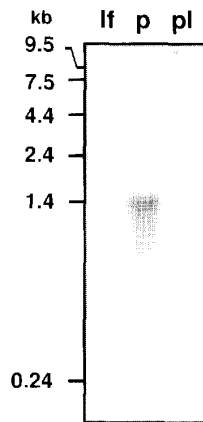


Fig. 2. Northern blot of barley pollen cDNA. Ten μ g of total RNA extracted from leaves (lane lf), mature pollen (lane p), and pistils (lane pl) was probed with ^{32} P-cDNA labelled by random hexanucleotide priming. **Methods:** Five grams of pollen were ground by hand in a mortar and pestle chilled on dry ice. Pulverized pollen was placed in guanidinium iso-thiocyanate buffer (Chirgwin et al., 1979) and macerated with a Polytron[®] tissue homogenizer for 30 s at 4°C. Cell debris was pelleted by centrifugation at $12\,000 \times g$ for 20 min and the resulting supernatant subjected to cesium chloride buoyant density ultracentrifugation (Chirgwin et al., 1979). Barley pollen poly(A)⁺ RNA was affinity purified from total RNA by oligo(dT)-cellulose (Collaborative Research) chromatography (Aviv and Leder, 1972). Pistils and leaves were harvested and immediately frozen in liquid N₂. The tissue was placed in guanidinium iso-thiocyanate buffer, and was ground in a glass/Teflon homogenizer until cells were visibly disrupted (5–10 min). The mixture was then macerated with a Polytron for 60 s at 4°C and processed as above. RNAs were electrophoresed in 1.5% agarose gels under denaturing conditions (Alwine et al., 1977) and blotted onto Hybond-N[®] (Amersham Ltd.) nylon membranes. Radiolabelled probes were prepared by random hexanucleotide priming (Sambrook et al., 1989) of purified cDNA inserts. Hybridization between probe and RNA was allowed to proceed for 12 h at 65°C in $6 \times$ SSC, $5 \times$ Denhardt's solution (0.1% w/v each BSA, Ficoll, polyvinylpyrrolidone), $100 \mu\text{g ml}^{-1}$ heat-denatured, sheared *E. coli* chromosomal DNA, and 0.1% (w/v) SDS. Final post-hybridization wash was with $0.1 \times$ SSC, 0.1% SDS for 15 min at 65°C. Autoradiography was for 12 h.

highly abundant 65-kDa protein, whereas controls (*E. coli* alone, non-IPTG-induced transformants, and *E. coli* containing hollow vector controls) did not contain the 65-kDa protein. The identity of the 65-kDa protein as an MBP fusion was further verified by immunodetection with anti-MBP monoclonal antibody (New England Biolabs). Crude protein extracts from these transformants were loaded onto a 2-cm diameter column packed with 30 ml of amylose resin (New England Biolabs). All other *E. coli* proteins were eluted from the column, while the MBP::974 fusion was retained. Purified fractions of MBP::974 fusion protein were recovered from the column by addition of 20 mM maltose.

2.5. Preparation of anti-974 polyclonal antibody

Mice were immunized with MBP::974 protein according to Harlow and Lane (1988). Primary intraperitoneal immunizations of 50 μ g of purified MBP::974 protein in 250 μ l of Freund's adjuvant were performed on six 8-week-old female BALB/c mice. At intervals, mice were boosted with 25 μ g of purified MBP::974 protein alone. Tail bleeds were performed 10 days following each boost, from which 200–400 μ l of serum were obtained and pooled. Serum was titred and used at a final dilution of 1:10 000 in TTBS. Resulting anti-MBP::974 polyclonal antibody could identify the MBP::974 fusion protein in crude *E. coli* extracts at a titre of 1:5000. Anti-MBP::974 antibody also identified purified MBP::974 protein and a 30-kDa protein in mature barley pollen extracts. Western blots of pollen, leaf, root, seed, germinated seed, and pistil proteins, when immunodetected with anti-MBP, indicated that MBP was not expressed by barley (not shown). Therefore, anti-MBP::974 was considered suitable to assay Hor v 9 protein expression in developing barley tissues. For simplicity, this antibody was referred to as anti-974.

Fig. 1. Nucleotide sequence and ORF of Hor v 9 cDNA hvp9742. The putative open reading frame is indicated by capitalized nucleotides grouped in codon triplets. The amino acid residues coded are indicated below each triplet using the three-letter code and are italicized. Nucleotide and amino acid sequences are numbered on the left; the numbering of amino acid residues begins at position –27 to indicate the signal peptide. The first amino acid of the mature polypeptide is double underlined. A polyadenylation signal sequence in the 3'-untranslated region is underlined. **Methods:** cDNA was synthesized from mature barley pollen poly(A)⁺ RNA in the presence of ^{35} S-dCTP using reagents and enzymes supplied by and according to Pharmacia LKB Biotechnology Inc. An *EcoRI*/*NotI* adaptor (also from Pharmacia) 14 nucleotides in length was blunt-end ligated to the cDNA. The cDNA was ligated to *EcoRI* pre-digested and phosphatased Lambda Zap II arms provided by Stratagene Cloning Systems. The ligated cDNA-Lambda Zap II was packaged using Gigapack II Plus (Stratagene) yielding approximately 100 000 recombinant plaques. The cDNA library was screened by plaque lift DNA hybridization procedures (Sambrook et al., 1989). Plaques were blotted onto Hybond-N (Amersham Ltd), and probed with purified c7.2 Kentucky bluegrass allergen ^{32} P-cDNA. Hybridization was at 65°C, in $6 \times$ SSC (900 mM NaCl, 90 mM sodium citrate, pH 7.5), $5 \times$ Denhardt's solution, $100 \mu\text{g ml}^{-1}$ heat-denatured, sheared *E. coli* chromosomal DNA, and 0.5% (w/v) SDS. Final washes were at 65°C, $0.2 \times$ SSC. DNA sequences were determined by dideoxy-nucleotide termination (Sanger et al., 1977) of DNA synthesized during Taq polymerase-based cycle sequencing (Murray, 1989) of plasmids (reagents and enzymes were supplied by Gibco-BRL). Sequencing was initiated from the pBluescript T7, T3, SK, KS, and M13-reverse priming sites found on both sides of the multiple cloning site of pBluescript. Subclones of hvp9742 were generated with *SacI* and *EcoRI* and were sequenced on opposite strands using these four primers.

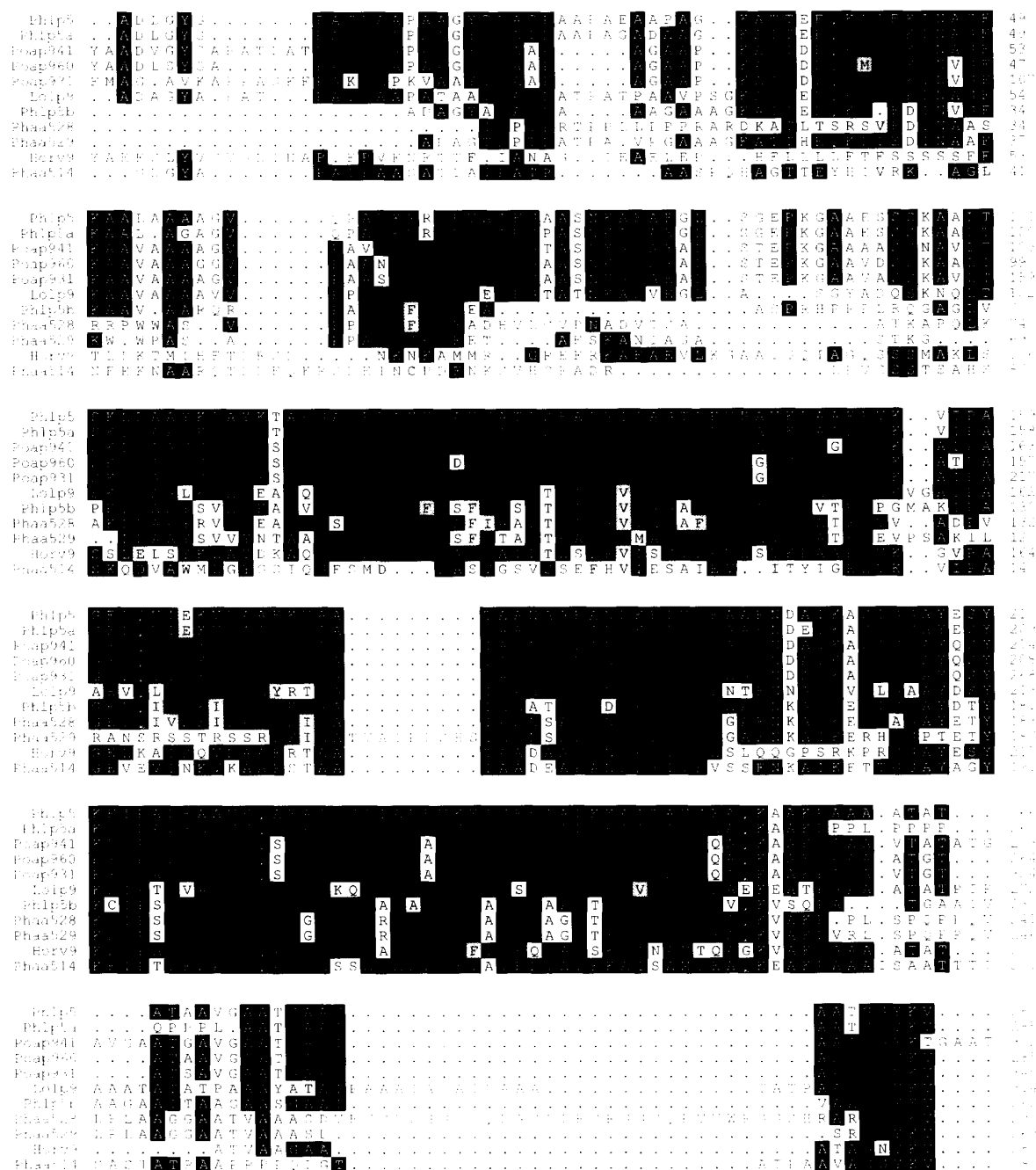


Fig. 3. Alignment of Hor v 9 with group 9 and group 5 pollen allergens. The amino acid sequence of Hor v 9 (GenBank accession no. U57845) was aligned with group 9 and group 5 pollen allergen sequences that were identified in the non-redundant sequence databases available at the National Center for Biotechnology Information world-wide-web server using BLASTA (Altschul et al., 1990). The identified sequences were Phl p 5 (Genbank accession no. X74735), Phl p 5a (Protein Resource Foundation accession no. 2023228A), Poa p 941 (KBG41 – Genbank accession no. M38343), Poa p 960 (KBG60 – Genbank accession no. M38344), Poa p 931 (KBG31 – Genbank accession no. M38342), Lol p 9 (Genbank accession no. L13083), Phl p 5b (Genbank accession no. Z27083), Pha a 528 (Pha a 5 clone 28 – NCBI accession no. 1246117), Pha a 529 (Pha a 5 clone 29 – NCBI accession no. 1246118) and Pha a 514 (Pha a 5 clone 14 – NCBI accession no. 1246119). Residues outlined in black boxes are identical, those in grey boxes are similar. The N-terminal 57 residues of Poa p 931 and the C-terminal 7 residues of Poa p 941 are not shown to save space.

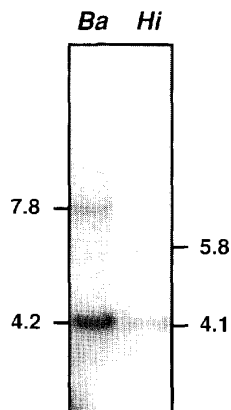


Fig. 4. Southern blot analysis of barley genomic DNA probed with Hor v 9 clone hvp97. Barley genomic DNA was digested with *Bam*HI (lane Ba) and *Hind*III (lane Hi), and was probed with clone 97. ^{32} P-cDNA was labelled by random hexanucleotide priming. DNA size markers are indicated on the left and right. **Methods:** Genomic DNA was prepared from leaves essentially as described by Murray and Thompson, 1980). Five grams (fresh weight) of leaf tissue were frozen under liquid nitrogen and stored at -70°C . Frozen leaves were ground in a mortar and pestle containing liquid nitrogen. Ten ml of 1% CTAB buffer (w/v), pre-warmed to 60°C , were added to the ground tissue and incubated 30 min at 60°C . Ten ml of chloroform (24:1 chloroform:isoamyl alcohol) were added, and the mixture was centrifuged at $1000 \times g$ for 10 min. The aqueous phase was recovered. A 0.4 volume of 5 M ammonium acetate was added, followed by 2 vol. of isopropanol. Precipitating DNA was centrifuged, recovered, and re-suspended in TE buffer. RNA was removed by treatment with RNase followed by repeated phenol-chloroform extractions. Genomic DNA was digested with restriction endonucleases and electrophoresed in 0.8% agarose gels. DNA was denatured and capillary blotted onto nylon (Hybond N-Plus, Amersham) membranes according to Sambrook et al. (1989). Hybridization between probe and DNA was allowed to proceed for 12 h at 65°C in $6 \times \text{SSC}$, $5 \times \text{Denhardt's solution}$, $100 \mu\text{g ml}^{-1}$ heat-denatured, sheared *E. coli* chromosomal DNA, and 0.5% (w/v) SDS. Final post-hybridization wash was with $0.5 \times \text{SSC}$, 0.1% SDS for 15 min at 65°C . Autoradiography was for 2 weeks.

2.6. Accumulation of Hor v 9 mRNA and protein in developing anthers

The expression pattern of the mRNA for the Hor v 9 gene was assessed by RNA gel blots of poly(A⁺) RNA prepared from leaves, pistils, anthers and mature pollen. Anthers were obtained on the basis of developmental stages (Astwood and Hill, 1995) as follows: stage 1 anthers contained unicellular microspores, stage 2 anthers contained bicellular pollen, stage 3 were non-dehiscent anthers containing tricellular pollen. Northern blots of the barley RNAs probed with clone hvp9742 showed that Hor v 9 was abundantly expressed in stage 3 anthers (Fig. 5A) and mature pollen (not shown). mRNA could also be detected in stage 2 anthers, but at approximately 50-fold lower levels compared to stage 3 anthers (Fig. 5B). It has been suggested that mature pollen is primed with dormant transcripts which will be

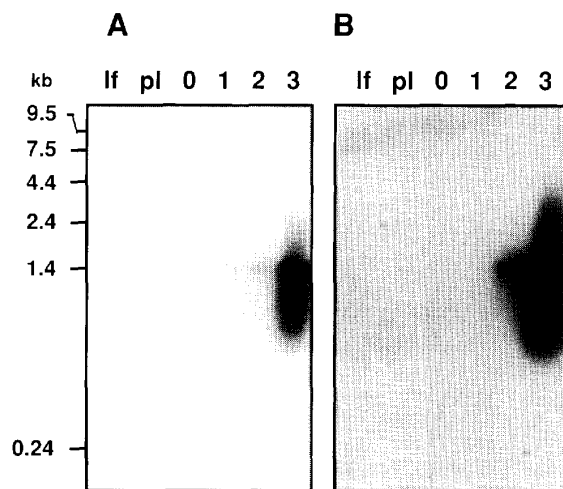


Fig. 5. Expression of Hor v 9 mRNA in development. Northern blot of barley poly(A⁺) RNAs expressed by different tissues and different times in development. One hundred ng of leaf (*lanxe lf*), pistil (*lane pl*), stage 1 anther (*lane 1*), stage 2 anther (*lane 2*), and stage 3 anther (*lane 3*) poly(A⁺)RNA were probed with ^{32} P-cDNA-labelled clone hvp9742 using methods described in Fig. 1. (A) Short exposure (3 h); (B) Long exposure (12 h).

translated during germination (Mascarenhas, 1993). Examples of genes expressed late in pollen development which continue to be expressed during germination include Zmc13 of maize (Hanson et al., 1989) and P2 of *Oenothera* (Brown and Crouch, 1990). Therefore, Hor v 9 mRNA may be required during pollen germination.

To assess the expression pattern of Hor v 9 protein, immunoblots of vegetative (seed, root and leaf) and reproductive (pistils, anthers and pollen) tissues were performed with anti-974. Anthers were staged as above, with the addition of stage L, which were dehiscent anthers containing mature pollen. Anti-974 antibody identified a 30-kDa protein accumulating from stage 3 anthers, through stage L anthers, to mature pollen; but not in other tissues (Fig. 6). One additional but weakly reactive protein of 57 kDa was also detected, especially in mature pollen. Hor v 9 protein synthesis in pollen development may represent an accumulation phase, and actual activity may not be required until germination. The expression pattern of the Hor v 9 protein was consistent with Northern assays, which suggested that the Hor v 9 was not translationally regulated.

2.7. Hor v 9 protein deposited on the pollen extracellular surface

To determine the location of Hor v 9 protein as expressed in pollen, whole mature pollen grains were fixed and immunodetected with anti-974 antibody.

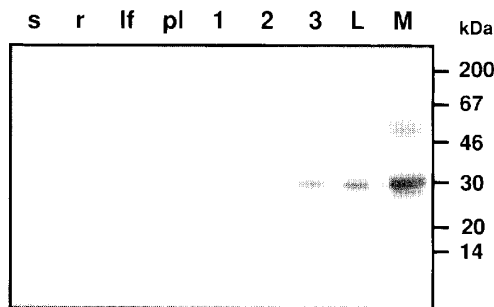


Fig. 6. Synthesis of Hor v 9 protein in development analyzed by Western blot of proteins expressed by different tissues at different times in anther development. Ten μ g of seed (lane *s*), root (lane *r*), leaf (lane *lf*), pistil (lane *pl*) stage 1 anthers (lane *1*), stage 2 anthers (lane *2*), stage 3 anthers (lane *3*), late stage 3 anthers (lane *L*) and mature pollen (lane *M*) proteins were immunodetected with anti-974 antibody. **Methods:** Proteins were extracted from developing anthers and mature pollen as follows: 100 staged anthers were harvested, frozen on a dry-ice/ethanol bath, freeze-dried, weighed and macerated with a glass/Teflon homogenizer in deionized distilled water until pollen grains were visibly disrupted, centrifuged for 15 min at $8000 \times g$, and the supernatant was recovered. Root, leaf and pistil tissues were extracted likewise. Protein content was determined by a micro-titre assay (Bradford, 1976). Protein extracts were loaded onto 15% SDS-PAGE gels according to Laemmli (1970) and electroblotted onto nitrocellulose according to Towbin et al., 1979). Blocking solution was 3% gelatin (w/v) in TBS (20 mM Tris, 500 mM NaCl, pH 7.5). For immunodetection, anti-974 serum was diluted 1:10 000 in TTBS (TBS plus 0.05% Tween-20, v/v) containing 1% gelatin (w/v). Antigen-antibody conjugates were visualized by reaction to alkaline phosphatase-linked goat anti-mouse IgG diluted 1:7500 in TTBS containing 1% gelatin (w/v). Color reaction of alkaline phosphatase was according to the manufacturers instructions (Bio-Rad Laboratories).

Pollen grains treated with alkaline phosphatase-linked anti-IgG, when observed by standard light microscopy, were optically transparent and white in colour. Grains treated with both anti-974 antibody and alkaline-linked anti-IgG antibody, when observed by standard light microscopy, were opaque (Fig. 7A) and darker in colour (Fig. 7B). Hor v 9 was readily apparent on the surface of the pollen grains.

It has been previously observed that the signal peptide of Lol p 9 may direct the protein to the amyloplast (Singh et al., 1991). Staff et al. (1990) have shown that many group 1 allergens of *Lolium perenne* were located in the exine of mature pollen. The current model accounting for the allergenicity of grass and other major pollen allergens suggests that allergens are rapidly released during imbibition upon deposition on the nasal mucosal membrane (Ong et al., 1996). This model is based, in part, on the observation that many major pollen allergens are released in a matter of minutes, whereas non-allergens like HSP70 are not released (Vrtala et al., 1993a). Pollen allergen release may occur via microchannels in the extracellular matrix of the

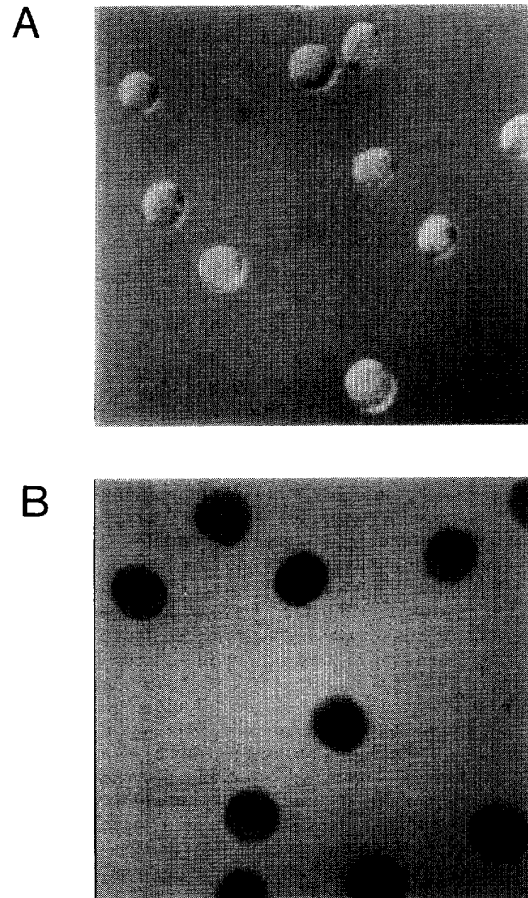


Fig. 7. Immunodetection of Hor v 9 protein on whole pollen grains. **Methods:** Mature pollen grains were placed directly into fixative containing 2% paraformaldehyde, 0.5% glutaraldehyde, 30 mM Pipes/pH 7.5, 10 mM CaCl_2 (Pogson et al., 1989) for 8 h at room temperature. Fixed pollen grains were washed three times in TTBS and suspended in a blocking solution (3% gelatin w/v in TTBS) for 30 min. Pollen was washed again 3 times in TTBS and suspended in a solution of antibody overnight. Pollen was washed three times with TTBS and suspended in secondary antibody (anti-mouse IgG) for 2 h. Pollen was pelleted and washed three times in TTBS, and once in TBS prior to alkaline phosphatase color development. As controls, fixed pollen was incubated in no antibody, primary antibody (anti-974) only, and secondary antibody only. All controls were subjected to alkaline phosphatase color development. Pollen grains were observed by standard light microscopy throughout the various treatments. (A) Control with pre-immune serum; (B) Immunodetection with anti-974.

pollen grain (Baraniuk et al., 1992). The localization of Hor v 9 to the surface of the glutaraldehyde/paraformaldehyde fixed barley pollen grain suggests that our method has caught Hor v 9 in the act of release from the grain. Indeed, we have observed that Hor v 9 protein is released from non-fixed pollen during nitrocellulose tissue-print experiments (not shown).

3. Conclusions

- (1) Several homologues of *Poa p 9* have been identified in a barley pollen cDNA library. All were expressed abundantly in pollen, and one, hvp9742, represented a full-length cDNA for *Hor v 9*. The cDNA encoded a 313-amino acid polypeptide including a putative 27-amino acid signal peptide, a potential glycosylation site, and a possible transmembrane sequence. The mature polypeptide was predicted to be 30.1 kDa, and was verified by using anti-974 antibody to detect the 30-kDa protein in barley pollen. The sequence of hvp9742 was greater than 67% similar to group 9 homologues of Kentucky bluegrass, and was named *Hor v 9*. Barley pollen clones hvp97, 971, and 974 were partial cDNAs contained within the full length hvp9742.
- (2) The mRNA and protein for *Hor v 9* clone hvp9742 accumulated maximally in mature pollen, and were not detected in pistil, leaf or root tissues.
- (3) Southern blotting experiments showed that one or two copies of the *Hor v 9* clone hvp9742 were present in the barley genome; and that there could be additional copies of group 9 genes in barley.
- (4) Group 9 genes in barley (*Hor v 9*), Kentucky bluegrass (*Poa p 9*) ryegrass (*Lol p 9*), Timothy (*Phl p 5*) and canary grass (*Pha a 5*) were highly conserved in the carboxyl-terminal two-thirds of the ORF. Computer modelling predicted the presence of a transmembrane motif in this conserved region.
- (5) *Hor v 9* localized to the surface of the pollen grain, which was consistent with the behavior of other group 9, group 5 and other pollen allergens which are rapidly released during imbibition.

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