

Proteomic analysis reveals developmentally expressed rice homologues of grass group II pollen allergens

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Abstract. Three isoallergens of *Oryza sativa*, homologues of grass group II pollen allergens, were identified from rice and characterised by proteome and immunochemical analyses. The *N*-terminal amino acid sequence profiles of three proteins on a 2-dimensional electrophoresis (2-DE) gel of rice pollen proteins matched 100% to the protein sequences encoded by three rice expressed sequence tags (ESTs). The deduced protein sequences from these ESTs share sequence identities of 41–43% with the protein sequences of the group II pollen allergens of different grasses, and sequence identity of 39% with the *C*-terminal portion of rice group I pollen allergens. Signal peptide sequences, which are similar to the leader peptides of other major pollen allergens, are also present in the deduced amino acid sequences. Polyclonal antibodies, produced in rabbits using *Oryza sativa* proteins purified by 2-DE, were used to investigate the developmental-stage- and tissue-specific expression of *Oryza sativa* by immunochemical analysis. Results of immunochemical experiments show that *Oryza sativa* proteins are expressed only at the late stage of pollen development and they do not have cross-reactivity with group II pollen allergens from some other common grasses.

Keywords: antibody, *N*-terminal sequencing, *Oryza sativa*, western blot, 2-dimensional gel electrophoresis.

Introduction

Hayfever and seasonal allergic asthma resulting from pollen allergies can affect a substantial proportion (20–25%) of the human population living in temperate regions (Wuthrich 1989; Griffith *et al.* 1991). Several groups of allergens from different grass pollens have already been isolated and characterised (for review see Ticha *et al.* 2002). Among them the group I pollen allergens, also known as β -expansins, are 28–34-kDa glycoproteins to which 85–90% of grass pollen allergic patients are sensitised (Freidhoff *et al.* 1986). Another group of well-characterised pollen allergens are group II allergens. One of the extensively investigated group II pollen allergens of the grass family is Lol p II from the perennial rye grass (*Lolium perenne*) (Ansari *et al.* 1987, 1989a). Lol p II is a group of acidic proteins (pH 5.0–5.3) with a molecular weight of 11 kDa, towards which 45% of grass pollen allergic patients are sensitive (Freidhoff *et al.* 1986). In the pollen of rye grass the Lol pII exists as immunologically indistinguishable isoallergens with isoelectric points in the range of 5.0–5.3. Group II allergens have also been identified and characterised from orchard grass (*Dactylis glomerata*) (Roberts *et al.* 1993), timothy grass (*Phleum pratense*)

(Dolecek *et al.* 1993), Bermuda grass (*Cynodon dactylon*), Kentucky blue grass (*Poa pratensis*), and wheat (*Triticum aestivum*) (NCBI accession numbers are CAA10346, CAA10348 and CAA10349, respectively. Submitted by Sturaro and Viotti 1998).

Rice (*Oryza sativa*), a member of the grass subfamily Ehrhartoideae, is cultivated as a major cereal crop in most populated Asian regions. Airborne rice pollens originating from rice fields can be a trigger of seasonal hayfever for the rice farmers and people living near rice-growing regions, as protein extracts of rice pollen have shown cross-allergenicity with other grasses (Kimura *et al.* 1969; Smith *et al.* 1994a). It has also been reported that there is a significant correlation between airborne rice pollen counts and asthma episodes in children during the flowering season of rice plants in Japan (Matsumura *et al.* 1969). In Taiwan, skin prick tests of asthmatic patients using rice pollen extracts showed that 9.3% of the asthmatic patients had a positive reaction. In addition, immunoblotting studies using positive serum from asthma patients revealed three major allergens with molecular weights of 16, 26 and 32 kDa, respectively (Tsai *et al.* 1990). *Oryza sativa* 1, a gene coding for the group I allergen of rice pollen, has been cloned and

Abbreviations used: BSA, bovine serum albumin; DBH, days before heading; EST, expressed sequence tag; IPG, immobilised pH gradient; ORF, open reading frame; PVDF, polyvinylidene difluoride; 2-DE, 2-dimensional electrophoresis.

characterised by molecular biological and immunochemical methods (Xu *et al.* 1995). A comparison of the deduced amino acid sequence of Ory s 1 with those of group I allergens from Bermuda and rye grasses revealed that they have a significant level of sequence similarities. Western blot analysis also demonstrated that the Ory s 1-encoded protein shares a high level of cross-allergenicity with other grasses. These studies suggested that rice pollen allergens could be major triggers of hayfever and seasonal asthma. However, rice pollen allergens were poorly characterised compared with their counterparts in other grasses. One reason for this situation could be the low prevalence of rice pollen as an aeroallergen in metropolitan areas.

So far there has been no report of isolation and characterisation of group II allergens from the pollen of rice. In this paper, we report the identification of three isoforms of group II allergens of rice pollen by 2-DE based proteome analysis. We also studied the tissue-specific expression of rice group II pollen allergens and cross antigenicity with other grasses using a polyclonal antibody raised against Ory s 2, group II allergens from rice.

Materials and methods

Reagents

All the chemicals were purchased from Sigma (St Louis, MO) unless stated otherwise.

Plant material

The Australian rice cultivar, Doongara, was grown in a glasshouse as previously described (Imin *et al.* 2001). Mature pollen grains were isolated from fresh heading-stage panicles according to Schrauwen *et al.* with some modifications (Schrauwen *et al.* 1990). In order to obtain a near-synchronous pollen population, only the top three primary branches were used in our experiments. The panicles were homogenised for 1 min in iced 0.3 M mannitol using an electric blender (Quickie-Super-7, Matsushita Elec. Ltd, Osaka, Japan). The suspension was filtered through a nylon sieve (70 µm) and pollen grains were pelleted by centrifugation at low speed (250 g, 1 min). The pellet was washed twice in iced 0.3 M mannitol. After the final wash, the isolated pollen grains were kept at -80°C until use. The heading stage rice anthers, palea, lemma, and stigma were collected by manual dissection of spikelets with forceps and a scalpel. The top flag leaves were also collected from the same plants. All the plant materials were kept at -80°C until use. The pollen of Bermuda grass (*Cynodon dactylon*), maize (*Zea mays*), Kentucky blue grass (*Poa pratensis*), rye grass (*Lolium perenne*), timothy grass (*Phleum pratense*) and wheat (*Triticum aestivum*) were obtained from Greer Laboratories (NC, USA).

Two-dimensional gel electrophoresis and peptide mass fingerprinting analysis

The extraction of proteins from rice pollen and anthers for two-dimensional electrophoresis (2-DE), 2-DE analysis and gel staining was performed as previously described (Natera *et al.* 2000; Imin *et al.* 2001). Isoelectric focusing was performed using 11- or 18-cm immobilised pH gradient (IPG) strips (linear pH 4–7, Amersham Biosciences, Uppsala, Sweden) and the second dimensional separation was carried out with 12–14% ExcelGel-SDS gels (Amersham Biosciences) on a Multiphor II horizontal electrophoresis unit.

N-terminal sequencing and database search

The proteins separated by 2-DE were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Immuno-Blot™, Bio-Rad, Hercules, CA) as previously described (Guerreiro *et al.* 1997). The PVDF membranes were stained with 0.1% (w/v) Coomassie brilliant blue R-250 (Bio-Rad) in 40% (v/v) methanol for 5 min and then destained in a solution containing 50% (v/v) methanol, 5% (v/v) acetic acid. Selected spots were excised from the air-dried membranes and the N-terminal sequencing was done on a PROCISE 494-01 sequencer system (Perkin-Elmer Applied Biosystems, Foster City, CA). N-terminal amino acid sequence homology comparison used the FASTA program (<http://www.ebi.ac.uk/fasta33/> posted 14 March 2003, verified 10 July 2003) to screen the SWISS-PROT protein database, and a tBLASTn search (Altschul *et al.* 1997) of rice EST sequences (Rice Gene Index, available at <http://www.tigr.org/tdb/tgi/ogi/> posted 21 April 2003, verified 10 July 2003). Translated protein sequences of the matching open reading frames (ORFs) were analysed for signal peptide cleavage sites using the SignalP program (Nielsen *et al.* 1997). A conserved domain search of the predicted protein sequence was done using the Pfam 7.6 database available at <http://pfam.wustl.edu/> (posted 2 June 2003, verified 10 July 2003) and ScanProsite (Gattiker *et al.* 2002) available at <http://www.expasy.ch/tools/scanprosite/> (posted 12 November 2003, verified 10 July 2003). Multiple sequence alignments were performed using ClustalX program (Thompson *et al.* 1997).

Antibody production

Polyclonal antibody production from 2-DE-purified proteins was performed as previously described (Drenckhahn *et al.* 1993), with some modifications. Briefly, the pH 5–6 portions of multiple isoelectrically focused 18-cm IPG strips (pH 4–7), which contained the proteins of interest, were separated in the second dimension as described above. Then, the proteins were electroblotted onto nitrocellulose membrane (Amersham Biosciences) as described above, and the transferred protein spots were visualised by staining the membranes in 0.1% (w/v) Ponceau S in 1% (v/v) acetic acid for 1 min. The membrane-bound protein spots were excised from the membranes, destained in deionised water and homogenised in liquid nitrogen until powdered. The homogenised membrane powder containing approximately 50 µg of gel-purified Ory s 2 protein was suspended in 2 mL of PBS (pH 7.6) and injected subcutaneously into New Zealand White rabbits. Two booster injections were administered three and six weeks after the first injection. Blood was withdrawn from an ear vein every two weeks and the antibody titre was checked by western blot analysis. Prior to immunisation, pre-immune serum was taken as a control.

Immunoblotting

To study the tissue- and stage-specific expression of Ory s 2-B/C, plant materials including rice anthers collected at different stages, mature pollen, leaf, stigma, shoot, root, and palea and lemma were homogenised in a liquid-nitrogen-cooled mortar and pestle before adding SDS sample buffer (63 mM Tris-HCl, 10% glycerol, 2% SDS, pH 6.8). The extracts were boiled, centrifuged (12000 g) to remove insoluble materials, and the supernatant was collected. The protein concentration of the diluted supernatant was determined by Bradford dye-binding assay (Bio-Rad, Hercules, CA). The samples, containing 60 µg of total protein, were fractionated using Novex precast 10–20% Tricine gels (Invitrogen, Carlsbad, CA). For 2-DE immunoblot of pollen proteins, the sample was fractionated using 11-cm IPG strips (pH 4–7) and 12–14% ExcelGel-SDS gels. The proteins separated by SDS-PAGE and 2-DE were transferred onto PVDF membrane (Bio-Rad) using the same method as described above. After the protein transfer, non-specific binding sites on the membranes were blocked by incubating the membranes in 7% skim milk powder in TBS-T

[20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% (v/v) Tween-20] overnight at 4°C. The blots were incubated for 60 min at room temperature with antiserum (1:3000 dilution in TBS-T) raised against Ory s 2-A and Ory s 2-B/C and developed with an ECL-Plus western blot detection kit (Amersham Biosciences) according to the manufacturer's instructions. Horseradish-peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) was used as a secondary antibody.

Immunolocalisation

Rice anthers from different developmental stages were briefly degassed in a fixing solution containing 4% (v/v) formaldehyde in phosphate buffer (0.1 M NaPO₄, pH 7.2) and incubated at room temperature for 2 h. After three washes with phosphate buffer, the material was dehydrated through a graded ethanol series (60%, 70%, 90% and 100%). The material was then infiltrated and embedded in hydroxyethyl methacrylate resin in ultra thin polymerase chain reaction tubes using a Leica Historesin embedding kit (Leica Instruments, Wetzlar, Germany) according to manufacturer's instruction. Semi-thin sections (2–4 µm) were cut with a glass knife on a Reichert-Jung ultramicrotome (Leica Instruments) and were mounted on poly-L-lysine-coated glass slides (Sigma). Non-specific binding sites of the sections were blocked by incubating the sections in 2% (w/v) cold fish gelatine in TBS-T for 30 min at room temperature. The sections were incubated with anti-Ory s 2-B/C antibody diluted to 1:50 with TBS-T containing 0.1% (w/v) bovine serum albumin (BSA) overnight at 4°C in a moist chamber. After washing three times with TBS-T-BSA solution for 30 min, the sections were incubated with anti-rabbit IgG-alkaline phosphatase conjugate (1:100 dilution; Boehringer Mannheim, Mannheim, Germany) in a TBS-T-BSA solution for 60 min in a moist chamber at room temperature. The sections were washed three times with TBS-T-BSA solution for 45 min and the colour reaction was performed using NBT/BCIP stock

solution (Boehringer Mannheim) according to the manufacturer's instruction. Control sections were treated identically except that pre-immune rabbit serum was substituted for Ory s 2-B/C antibody in the primary antibody incubation step. The slides were mounted with Permount (Fisher Scientific, Pittsburgh, PA) and observed under Nikon Optiphot light microscope. Photographs were taken on Fujichrome 400 colour slide film using a Nikon FX-35 A camera.

Results

Identification of Ory s 2 isoforms by N-terminal sequencing and database searching

A protein extract of rice pollen at the panicle-heading stage was prepared and subjected to 2-DE analysis. The separation of pollen proteins across the pH range of 4–7 is shown in Fig. 1. Two highly abundant, low molecular weight protein spots, with the same molecular mass of 11 kDa and different pIs, were excised from the PVDF blot and the N-terminal amino acid sequences were determined by Edman degradation. Unequivocal sequence data of 15 amino acid residues was obtained for protein spot t1-76 by N-terminal micro sequencing (Table 1). Clear sequence data were obtained for the first 15 amino acid residues for spot t1-17. However, a polymorphism was observed at residue 14 with serine and threonine both being detected in a 0.5:0.5 ratio. Figure 2 shows the chromatogram of the residue 14 obtained from the N-terminal sequencing of spot t1-17. Based on this data we concluded that two distinct proteins with very similar molecular weights and pIs are present at the position of

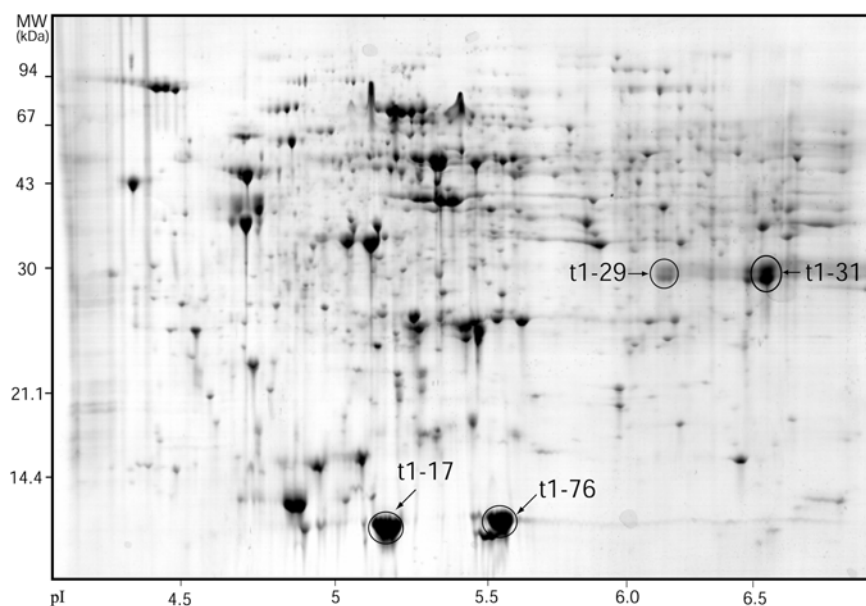


Fig. 1. 2-DE map of proteins extracted from the mature pollen of rice cultivar Doongara. For the first dimension, 1 mg of extracted pollen proteins was loaded on an IPG strip (linear pH 4–7, 18 cm). For the second dimension, a 12–14% SDS-PAGE gel was used. Proteins were visualised by colloidal coomassie staining. The molecular weight markers are denoted on the left and pI is indicated at the bottom. The protein spots t1-17 and t1-76 were subjected to N-terminal microsequencing analysis and the protein spots t1-29 and t1-31 were identified as isoforms of group I allergens (Ory s 1) in our previous report (Kerim *et al.* 2003).

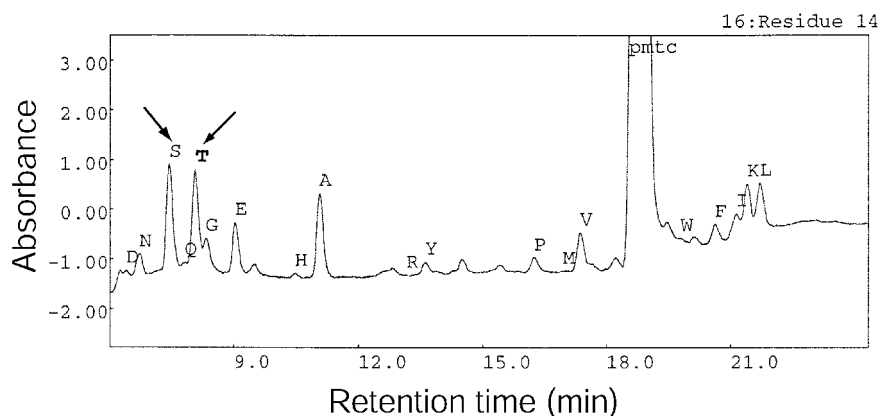


Fig. 2. Chromatogram of the residue 14 obtained from the *N*-terminal micro-sequencing of spot t1-17. The single letter amino acid abbreviations above the peaks indicated by arrows are corresponding to two amino acids, Serine and Threonine, obtained for residue 14 of spot t1-17.

protein spot t1-17. Therefore, two sets of *N*-terminal amino acid sequences, as shown in Table 1, were used for sequence homology searching for spot t1-17.

FASTA searches of the SWISS-PROT non-redundant protein database using these three sets of experimental *N*-terminal amino acid sequences did not yield any significant matches. BLAST searches of the GenBank protein database for short, nearly-exact matches revealed that the two *N*-terminal sequences derived from spot t1-17 exhibited 100% sequence identities in 15 amino acids to two hypothetical rice proteins, OSJNBa0050F15.10 (CAD40510) and OSJNBa0050F15.9 (CAD40509), predicted from genomic sequencing (Table 1). Further tBLASTn searching of the rice gene index of The Institute for Genomic Research revealed that each of the three *N*-terminal sequences displayed 100% identity over 15 amino acid residues to the ORF products of three EST sequences (Table 1). Each of the matching ORFs encodes a polypeptide of 117 amino acids with a molecular weight of about 12 kDa. SignalP analysis predicted signal peptide sequences of 23 amino acids in all three deduced protein sequences (Fig. 3). Furthermore, the cleavage sites of the signal peptides are predicted to be located immediately before the start of the experimentally determined *N*-terminal sequences. The calculated molecular weights and pIs of the deduced mature proteins are in agreement with those observed experimentally on 2-DE gels

(Table 1). Alignment of the deduced protein sequences using the ClustalX program showed that these three sequences share 86.3% (between Ory s 2-A and Ory s 2-C), 90.6% (between Ory s 2-A and Ory s 2-B), and 93.2% (between Ory s 2-B and Ory s 2-C) amino acid sequence identities (Fig. 3). The results of conserved-domain searching show that all three predicted protein sequences contain a conserved pollen allergen domain (PFAM accession PF01357). The conserved domain matches to amino acids 26–102 in the deduced protein sequences. They also contain an expansin cellulose-binding domain (EXPANSIN-CBD, PROSITE accession PS50843), which matches to amino acid residues 47–113. Both of the domains are characteristic of group I and group II pollen allergens. Figure 3 demonstrates the alignment of the deduced protein sequences with amino acid sequences of known group II pollen allergens. The Ory s 2 isoforms share comparable amino acid sequence identities (41.7–43.5%) with group II pollen allergen proteins of orchard grass, Bermuda grass, timothy grass, wheat, velvet grass and Kentucky blue grass. Sequence alignment analysis also indicates 39%, 41% and 38% sequence identities between the deduced protein sequences of Ory s 2-A, Ory s 2-B and Ory 2-C, and the C-terminal portion (starts from amino acid residue 168–267) of rice group I pollen allergen (Ory s 1), respectively.

Table 1. Proteins analysed by *N*-terminal micro-sequencing and database searching

Spot ID.	Mr/pI ^A	<i>N</i> -terminal sequence	Homology (% identity)	NCBI accession	MW/pI ^B	Signal peptide	TIGR accession
t1-76	11/5.6	TEVTFKVGEGSSGKS	100%	—	10.0/5.45	Yes	TC90336
t1-17	11/5.2	TELTFKVAEGSSASS	100%	CAD40510	10.1/5.12	Yes	TC83171
		TELTFKVAEGSSATS	100%	CAD40509	10.1/5.13	Yes	NP497980

^AThe observed molecular mass and pI of the protein spots. ^BThe predicted molecular mass and isoelectric point of the matched peptides calculated from the ORFs after excluding the predicted signal peptide sequences.

Fig. 3. Amino acid sequence alignment of Ory s 2-A, B and C with group II pollen allergens of other common grasses. Multiple sequence alignment was performed using ClustalX program (Thompson 1997). The accession numbers and sources of the proteins are: Ory s 2-A (SWISS-PROT accession number P83466); Ory s 2-B (CAD40510); Ory s 2-C (CAD40509); Hol l 2 (CAA10347) of velvet grass; Lol p II (CAA10350) of rye grass; Poa p 2 (CAA10348) of Kentucky bluegrass; Tri a 2 (CAA10349) of wheat; Phl p 2 (P43214) of timothy grass; Dac g 2 (CAA10345.1) of orchard grass and Cyn d 2 (CAA10346) of Bermuda grass. The symbols on the top of each column show the homology among the three isoforms of Ory s 2 while the symbols on the bottom show the homology among all the sequences in the alignment. * Indicates that the residues in that column are identical in all sequences in the alignment; ':' and '.' indicate that the residues in that column are conserved and semi-conserved, respectively. The experimentally determined N-terminal sequences of Ory s 2 proteins are underlined. The bar on the top of the columns shows the location of the pollen allergen domain (PFAM accession PF01357) in the alignment.

is any significant immunological cross-reactivity between Ory s 2 and group II pollen allergens of other grasses. The western immunoblot results showed that the polyclonal antibody did not bind to any protein components, within molecular weight range of 10–12 kDa (group II allergens), in protein extracts of six common grasses (Fig. 6). However, it bound to a band with a molecular weight of approximately 32 kDa in maize pollen extracts. Although there is a reasonably high level of sequence similarity between Ory s 2 and group II pollen allergens from other grasses, demonstrated by our sequence alignment analysis, no cross-reactivity of Ory s 2 to these allergens was detected.

Immunolocalisation of Ory s 2 in rice anthers

The 2 DE and immunoblot analysis results show that Ory s 2 proteins are expressed only in late-stage pollen and anthers. To confirm pollen-specific expression of Ory s 2, tissue localisation of Ory s 2-B/C in cross sections of rice anthers was

performed using immuno-light microscopy. The Ory s 2-B/C is exclusively localised in the cytoplasm and cell wall of mature pollen (Fig. 7A), but no colour reaction product was detected in nuclei region of pollen and anther wall tissues. In control experiments using pre-immune serum, the anther sections showed a low background colour (Fig. 7B).

Discussion

Type I allergy is a prevalent global medical problem. Among the most potent elicitors of type I allergy are group II/III allergens of grass pollen. The group II allergens have been identified and characterised from a number of common grasses. However, so far there is no report on identification and characterisation of group II/III pollen allergens of rice, a widely cultivated crop plant and a member of the grass family. The accumulated rice EST and genome sequences in the public databases already contain partial or complete sequences of genes coding for some rice allergens. These

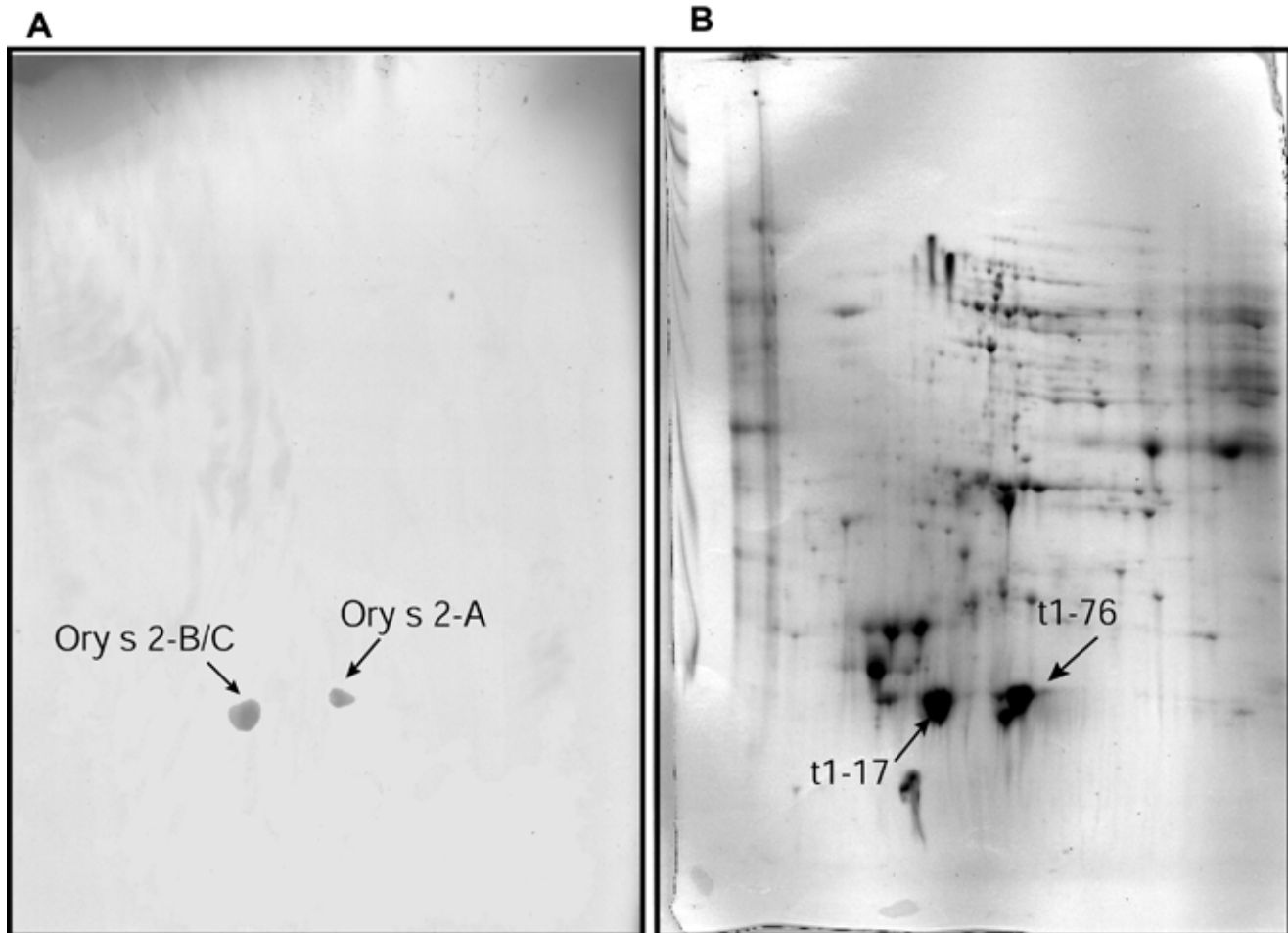


Fig. 4. 2-DE immunoblot analysis using anti-Ory s 2-B/C serum. Pollen protein extract (500 µg) was separated using a 11-cm IPG strip in first dimension and SDS-PAGE in second dimension. (A) The separated proteins were electroblotted onto PVDF membrane and probed with anti-Ory s 2-B/C serum with a chemiluminescence detection kit. The detection signals marked as Ory s 2-A and Ory s 2-B/C correspond to the protein spots t1-76 and t1-17 in Fig. 4B, respectively. (B) A replica gel of Fig. 4A was stained with colloidal coomassie stain and the protein spots corresponding to the detection signals in Fig. 4A are marked.

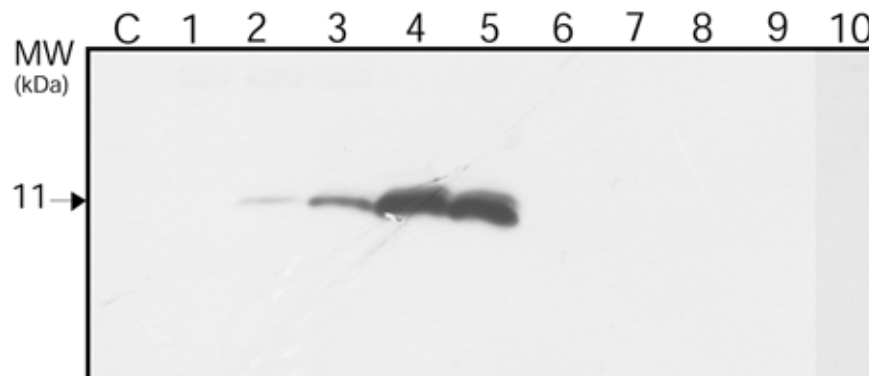


Fig. 5. Stage- and tissue-specific expression of Ory s 2 revealed by western immunoblot analysis. Total soluble proteins, which were extracted from rice pollen and anthers at different developmental stages and from different tissues of rice plant, were fractionated by SDS-PAGE and electroblotted onto PVDF membrane. The blot was probed using anti-Ory s 2-B/C antibody. Lane 1, anthers at 8 d before heading (DBH); lane 2, anthers at 5 DBH; lane 3, anthers at 3 DBH stage; lane 4, anthers at heading stage; lane 5, pollen at heading stage; lane 6, leaf; lane 7, stigma; lane 8, shoot; lane 9, root and lane 10, palea and lemma. Lane C, heading-stage pollen protein was probed with pre-immune serum as a negative control.

databases have made it easier to identify these allergens using proteome analysis techniques without using immunological screening and cloning procedures. We reported earlier during our proteome analysis of rice male gametophyte development, the peptide mass fingerprinting and *N*-terminal amino acid sequence profiles of three late-stage-specific proteins matched to the translation products of three unannotated EST sequences in public databases (Kerim *et al.* 2003). Since then, further investigations have been carried out to confirm the identities of the matching EST sequences.

On the basis of the sequence homology among deduced protein sequences of matching ORFs, it can be concluded that Ory s 2-A, Ory s 2-B and Ory s 2-C are homologous proteins. Cross-reactivity of the polyclonal antibodies to Ory s 2-A and Ory s 2-B/C protein spots on a 2-DE blot also supported this conclusion. The results of further bioinformatic analysis of matching ESTs in combination with immunochemical analysis provided several lines of evidence that the three proteins are rice homologues of group II pollen allergens of the grass family. First, the deduced amino acid

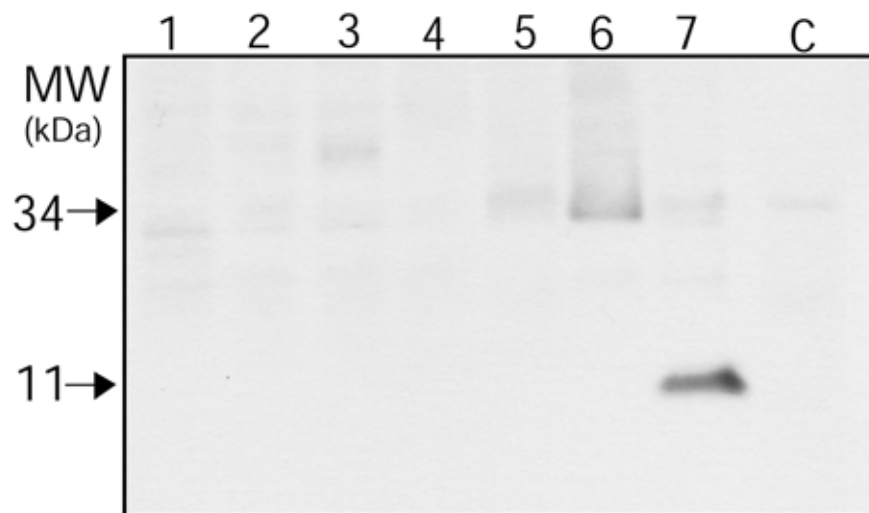


Fig. 6. Cross-reactivity of Ory s 2-B/C antibody with group II allergens of other grasses. Soluble pollen proteins from Bermuda grass (lane 1), Kentucky blue grass (lane 2), timothy grass, perennial rye grass (lane 4), wheat (lane 5), maize (lane 6) and rice (lane 7) were separated by SDS-PAGE and blotted onto PVDF membrane. The blot was probed with anti-Ory s 2-B/C antibody and detected with a chemiluminescence detection kit. Lane C, heading-stage pollen protein was probed with pre-immune serum as a negative control.

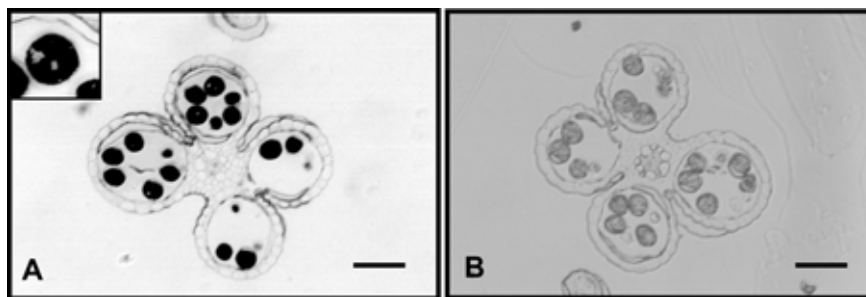


Fig. 7. Immuno-light microscopic localisation of Ory s 2 on cross-sections of heading-stage rice anthers. (A) The semi-thin anther cross-sections were probed with anti-Ory s 2-B/C antibody and detected with NBT/BCIP colour reaction substrate. The insert shows one pollen grain at higher magnification. The colour reaction product was detected in cytoplasm and cell wall of pollen grains. (B) Anther cross-sections were treated with pre-immune serum and detected in parallel with (A) (Bars = 100 µm).

sequences of the matching ESTs have signal peptide sequences 23 amino acids long. Furthermore, these signal peptide sequences share around 45% sequence identity with signal peptides predicted for other well-characterised group II allergens of other grasses. This means that Ory s 2 proteins are likely to be secreted, which is one of the common characteristics of pollen allergens. Second, the experimental and calculated molecular weights and pIs of the protein spots on 2-DE gels are in close agreement with molecular weights and pIs of group II pollen allergens from the grass family. They exist as immunologically indistinguishable multiple isoforms in the pI range of 5.0–5.4, as reported for Lol pII (Ansari *et al.* 1989b; Dolecek *et al.* 1993). Third, they share comparable sequence identities with other group II pollen allergens as demonstrated by sequence alignment analysis. At the same time, conserved pollen allergen domains and some other sequence motifs that are specific to grass pollen allergens are also present in Ory s 2 proteins. Fourth, the Ory s 2 sequences have 39% of amino acid sequence identity with the C-terminal portion of Ory s 1 (group I pollen allergen of rice). It was reported in rye grass that Lol pII also shares a similar level of sequence identity (33%) with the C-terminal part of Lol pI (Griffith *et al.* 1991). Finally, the temporal and spatial expression patterns of Ory s 2 proteins demonstrated by immunochemical experiments are consistent with the expression pattern of Ory s 1 and other group II allergens (Dolecek *et al.* 1993; Xu *et al.* 1995). The evidence presented indicates that these three proteins are the rice homologues of grass group II pollen allergens. Therefore, we designated them as Ory s 2-A, Ory s 2-B (OSJNBa0050F15.10) and Ory s 2-C (OSJNBa0050F15.9) according to the international allergen nomenclature system (King *et al.* 1994). The N-terminal amino acid sequence of Ory s 2-A protein was submitted to SWISS-PROT protein database with accession number P83466.

Early studies show that grass pollen allergens that belong to the same group but from different grass origins have

immunological cross-reactivity (Smith *et al.* 1994a, b). Furthermore, cross-reactivity of Ory s 1 with group I allergens from timothy, rye and orchard grasses has been demonstrated (Xu *et al.* 1995). In this report it is evident from the sequence alignment analysis that Ory s 2 proteins share a considerable level of sequence similarity with group II allergens from other grasses. Therefore, we expected some degree of cross-binding of anti-Ory s 2 antibodies to group II allergens of other common grasses. But our immunoblot analysis showed that polyclonal anti-Ory s 2 antibody of rice, which is a member of grass subfamily Ehrhartoideae, did not cross-bind to the counterparts from three other grass subfamilies including Pooideae (rye grass, Kentucky blue grass, timothy grass and wheat), Chloridoideae (Bermuda grass) and Panicoideae (maize). A possible explanation for this is that allergenic epitopes of group II allergens are not highly conserved among grass subfamilies. Another factor likely to contribute to a lack of cross-reactivity between group II allergens is the molecular size of group II allergens. The small molecular size of group II allergens may reduce the probability of conservation of common allergenic epitopes among group II allergens from different grass origins. Therefore, a lower level of cross-reactivity among the group II allergens than that among the group I allergens can be expected. However, the Ory s 2 antibody cross-bound to 32-kDa protein bands in maize pollen extracts. These proteins, presumably, are the group I allergens of maize and they have some cross-reactive epitopes with Ory s 2. The cross-reactivity between Ory s 2 and other grass group II pollen allergens needs to be further studied with IgE from patients allergic to rice pollen. Further study on immunological features of rice group II pollen allergens may lead to the application of these allergens for the immunotherapy of rice pollen allergy.

Although the allergic properties of grass pollen allergens have been studied intensively by immunologists, their native biological functions in plant reproductive developmental process are poorly understood. It was proposed that some

pollen allergens might play a role in pollen–stigma recognition during fertilisation (Knox 1973). Recently, an expansin activity of group I pollen allergens in grass cell walls was demonstrated and therefore the group I pollen allergens were classified as a β -expansin family (Cosgrove *et al.* 1997). However, the biochemical mechanism underlying expansin activities of group I allergen remains highly controversial as different biochemical activities were proposed to group I allergens (Li and Cosgrove 2001; Grobe *et al.* 2002). The considerable levels of sequence similarity and existence of some common functional domains between Ory s 2 and Ory s 1 raise the question of whether Ory s 2 also has expansin activity. So far there is no report about the physiological function of group II pollen allergens. The developmental-stage-specific expression and the high abundance of Ory s 2 proteins in mature pollen revealed by our investigation indicate that these proteins may have important functions in pollen development and pollen stigma interactions. Elucidation of the biological function of group II allergens using biochemical assays and molecular biology tools will contribute to a better understanding of molecular mechanisms underlying the male gametophyte developmental processes.

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