PROTEOME ANALYSIS OF MALE GAMETOPHYTE DEVELOPMENT IN RICE ANTHERS

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CHAPTER 5 IDENTIFICATION AND CHARACTERIZATION OF RICE HOMOLOGUES OF GRASS GROUP II POLLEN ALLERGENS

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SUMMARY

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 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 analyses. The results of immunochemical experiments demonstrated the late stage-specific expression patterns of *Oryza sativa* proteins, indicating the possible involvement of these proteins during the fertilization process. The *Oryza sativa* proteins do not cross-react with group II pollen allergens from some other common grasses.

5.1 INTRODUCTION

The grass family comprises about 9,000 species, and they are the most important group of plants both in ecological and economical terms. At the same time, they are also a main cause of allergic disease worldwide. Hayfever and seasonal allergic asthma resulting from the grass 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 Andersson and Lidholm 2003; Ticha *et al.* 2002). The allergenic properties of most of the identified grass pollen allergens are well studied. However, the precise biological functions of most of the grass pollen allergens are largely unknown (Andersson and Lidholm 2003).

One group of well-characterised grass pollen allergens is the group II allergens, which are a group of acidic small proteins (pH 5.0-5.3) with a molecular weight of 10-12 kDa (Freidhoff *et al.* 1986; Ansari *et al.* 1989b). Lol p II from the perennial rye grass is one of the extensively investigated group II pollen allergens, towards which 45% of grass pollen allergic patients are sensitive (Freidhoff, Ehrlich-Kautzky *et al.* 1986; Ansari *et al.* 1987; Ansari *et al.* 1989a;). Group II allergens have also been identified and characterised from orchard grass (Roberts *et al.* 1993a), timothy grass (Dolecek *et al.* 1993), Bermuda grass, Kentucky blue grass and wheat (NCBI accession numbers are CAA10346, CAA10348 and CAA10349, respectively. Submitted by Sturaro *et al.*).

Rice (*Oryza sativa*), a member of the grass subfamily Bambusoideae, 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 because protein extracts of rice pollen have shown cross-allergenicity with other grasses (Kimura *et al.* 1969; Smith *et al.* 1994a). It also has 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 32kDa, respectively (Tsai *et al.* 1990). *Ory s 1*, a gene coding for the group I allergen of rice pollen, has been cloned and characterised by molecular biological and immunochemical methods (Xu, Theerakulpisut *et al.* 1995a; Xu *et al.* 1999). Western blot analysis demonstrated that the *Ory s 1* encodes a protein which shares high level of cross allergenicity with other grasses. These studies suggested that rice pollen allergens could be major triggers of hay fever and seasonal asthma. However, the rice pollen allergens were poorly characterised compared to their counterparts in other grasses. One reason for this situation could be the low prevalence of rice pollen as an aeroallergen in metropolitan

areas. Although group II pollen allergens have been identified from several common grasses, so far there has been no report about isolation and characterisation of group II pollen allergens from rice.

As presented in the results of Chapter 4, the *N*-terminal amino acid sequences of the two differentially displayed rice anther protein spots were shown to match three TC sequences in the database. The deduced protein sequences of the TC sequences were shown to share some level of sequence homology with grass group II pollen allergens. Because of the stage specific regulation and the high abundance levels in the late pollen developmental stages, these proteins were chosen for further characterization using immunochemical and computer analysis tools. In this chapter, the tissue and stage-specific expression of rice group II pollen allergens, and their cross-antigenicity with other grasses were studied using polyclonal antibodies raised against the gel-purified proteins. At the same time, an attempt was made to study the interaction of the group II pollen allergens with rice pollen and style proteins using immunoprecipitation in order to identify the protein interaction networks, in which the group II allergens are involved. The main aim of this chapter was to further characterise the spatial and temporal expression of rice group II pollen allergens, and to examine their use as potential protein markers of specific pollen developmental stages.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

The Australian rice cultivar, Doongara, was grown in a glass house as described in Chapter 2. Mature pollen grains were isolated from fresh heading stage panicles according to Schrauwen *et al* (1990). with some modifications. In order to obtain a near synchronous pollen population, only the top three primary branches were used in the experiments. The panicles were homogenized 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 μ m) 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 anthers, palea, lemma, and stigmas were collected from the rice plants at heading stage by manual dissection of the spikelets using a pair of forceps and a scalpel. The top

flag leaves were also collected from the same plants for immunoblot analysis. All the plant materials were kept at -80°C until use. The pollens 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.)

5.2.2 Two-dimensional gel electrophoresis

The protein extraction from rice pollens and the 2-DE analysis was carried out as described in Chapter 2.

5.2.3 Computer analysis

The conserved domain search of the predicted protein sequence was done using the Pfam 7.6 database available at <http://pfam.wustl.edu/> and ScanProsite (Gattiker et al. 2002) available at <http://www.expasy.ch/tools/scanprosite/>. Multiple sequence alignments were performed using the ClustalX program (Thompson *et al.* 1997). The phylogenetic tree was constructed based on the distance matrix between sequence pairs using the MegAlign program of the DNASTAR software package (DNASTAR, Inc, Madison, USA)

5.2.4 Antibody production

Polyclonal antibody production using 2-DE purified proteins was done as previously described (Drenckhahn *et al.* 1993) with some modifications. Briefly, the pH 5 to 6 portions of multiple isoelectrically focused 18 cm IPG strips (pH 4 to 7), which contained the proteins of interest, were separated in the second dimension as described in section 2.3 of Chapter 2. Then, the proteins were electroblotted onto nitrocellulose membranes (Amersham Biosciences, Uppsala, Sweden), 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 proteins 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.

5.2.5 Immunoblotting

To study the tissue and stage specific expression of Ory s 2-A and Ory s 2-B/C, the plant materials including rice anthers collected at different stages, mature pollen, leaf, stigma, shoot, root and palea-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 x 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 proteins were fractionated using Novex precast 10-20% Tricine gels (Invitrogen, Carlsbad, CA). For 2-DE immunoblot of pollen proteins, the sample was fractionated using 11cm IPG strips (pH 4 to 7) and 12-14% ExcelGel-SDS gels. The proteins separated by SDS-PAGE and 2-DE were transferred onto PVDF membrane (Bio-Rad, Hercules, CA) using the same method as described in Chapter 2. 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 were developed with an ECL-Plus western blot detection kit (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. Horseradish peroxidase conjugated anti-rabbit IgG (Amersham Biosciences, Uppsala, Sweden) was used as a secondary antibody.

5.2.6 Immunolocalisation

Rice anthers from heading stage 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 two hours. After three washes with phosphate buffer, the material was dehydrated through a graded ethanol series (60%, 70%, 90% and 100%, v/v). Then, the material was infiltrated and embedded in hydroxyethyl methacrylate resin in ultra thin PCR tubes using a Leica Histo-resin embedding kit

(Leica Instruments, Wetzlar, Germany) according to the manufacturer's instructions. Semi-thin sections (2-4 mm) were cut with a glass knife on a Reichert-Jung ultramicrotome (Leica Instruments, Germany) and were mounted on poly-L-lysine-coated glass slides (Sigma, St.Louis, MO). The immunological detection was carried out as described before (Cho and Kende 1998) with some modifications. Nonspecific 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 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 instructions. Levamisole was added to the substrate at a final concentration of 1 mM to block the endogenous alkaline phosphatase activity of the anther sections. 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) and observed under Nikon Optiphot light microscope. Photographs were taken on Fujichrome 400 colour slide film using a Nikon FX-35 A camera.

5.2.7 Immunoprecipitation

Pooled plant material (200 mg of anthers and anthers plus style) was ground in a liquid nitrogen cooled mortar and pestle for 10 min and solubilised in 1 ml of protein extraction buffer (50mM Tris-HCl pH 7.6, 1 mM EDTA, 1mM DTT, 1x Protease inhibitor cocktail) on ice. The homogenate was centrifuged at 10000 g for 10 min at 4 °C and the supernatant was saved. The protein concentration of the supernatant was measured using Bradford assay. For each sample, 1 mg (250 µl in volume) of protein extract, 50 µl of anti Ory s-2 B/C antibody and 200 µl of immunoprecipitation buffer (50mM Tris-HCl pH 7.6, 100 mM NaCl, 0.5% (v/v) Nonidet-40, 1mM DTT, 1x Protease inhibitor cocktail) were mixed on ice. The mixture was incubated for 2 hours at 4°C on a rotating wheel. In the meantime, the protein A-agarose bead conjugate (Sigma,

St. Louis, MO) was pre-equilibrated by washing twice with immunoprecipitation buffer and suspended with twice the volume of immunoprecipitation buffer to make 50% protein A-agarose slurry. An aliquot of this slurry (100 μ l) was added to the reaction mix on ice, and the reaction mixture was incubated for further 30 min at 4°C on a rotating wheel. The beads were collected by centrifugation at 3000 x g for 2 min at 4°C, and washed 3 times with the immunoprecipitation buffer to exclude the unbound proteins. Proteins were eluted from the beads by boiling for 1 min in 50 μ l of SDS sample buffer (63 mM Tris-HCl, pH 6.8, 10% Glycerol, 2% SDS and 1mM DTT) and separated using Novex precast 10-20% Tricine gels. The proteins were visualized by colloidal coomassie staining.

5.3 RESULTS

5.3.1 2-DE analysis of pollen proteins

A proteome map of rice pollens (Figure 5.1), which were isolated from the heading stage panicles, was established by using exactly the same experimental conditions that were used for establishing the anther proteome maps (Chapter 4). The pollen proteome map was then compared to the anther proteome map at the heading stage (Figure 4.6, Chapter 4) to see if the protein spots t1-76 and t1-17 were anther or pollen specific. As shown in Figure 5.1, the protein spots t1-17 and t1-76 were present at the same gel locations where they were present on the anther proteome map at the heading stage (Figure 4.6, Chapter 4). Comparison of the spot volume showed that the volume of t1-76 and t1-17 on both proteome maps did not change significantly (less than 10%), indicating the pollen-specific expression of these proteins.

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

As presented in Chapter 4, the protein spots t1-17 and t1-76 were subjected to N-terminal microsequencing analysis. Unequivocal sequence data of 15 amino acid residues was obtained for protein spot t1-76 by N-terminal sequencing (Table 5.1). Clear sequence data was 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 to 0.5 ratio. Figure 5.2 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 a very similar molecular weight and pIs are present at the position of protein spot t1-17. The separation of the anther proteins using 24 cm IPG strips also revealed the presence of two protein spots on the location of the spot t1-17 (data not shown). Therefore, two sets of N-terminal amino acid sequences as shown in Table 1 were used for the 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 exact matches revealed that the two N-terminal sequences derived from spot t1-17 exhibited 100% sequence identities in 15 amino acid to two hypothetical rice proteins, OSJNBa0050F15.10 (CAD40510) and OSJNBa0050F15.9 (CAD40509), predicted from genomic sequencing (Table 1). Further tBLASTn searching of TIGR rice gene index revealed that each of the three N-terminal sequences displayed 100% identity over 15 amino acid residues to the ORF products of three TC sequences (Table 5.1). Each of the matching ORFs encodes for a polypeptide of 117 amino acids with a molecular weight of about 12 kDa. SignalP analysis predicted 23 amino acid long signal peptide sequences in all three deduced protein sequences (Figure 5.3). 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 close agreement with those experimentally observed on 2-DE gels. The homology searching of the NCBI protein database using the translated protein sequences revealed that all three sequences share some degree of sequence identities with the group II pollen allergens from various grasses. Therefore, they were designated as Ory s 2-A (t1-76) and Ory s 2-B/C (t1-17).

5.3.3 Sequence analysis of the matching ORFs

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 with each other (Figure 5.3). The results of conserved domain searching show that all three predicted protein sequences contain a conserved pollen allergen domain (PFAM accession PF01357), which matches to amino acid residues that starts from 26 to 102 in the deduced protein sequences. They also contain

Figure 5.1. 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 micro sequencing analysis and the protein spots t1-29 and t1-31 were identified as isoforms of rice group I pollen allergens (β -expansins) in Chapter 4.

Figure 5.2. 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.

Table 5.1. Proteins analysed by *N*-terminal microsequencing and database searching

Spot I.D.	Mr/pl ^{a)}	<i>N</i> -terminal Sequence	Homology (% identity)	NCBI accession	MW/pl ^{b)}	Signal peptide	TIGR accession
t1-76	11/5.6	TEVTFKVGEGSSGKS	100%		10.0 / 5.45	Yes	TC114263
t1-17	11/5.2	TELTFKVAEGSSASS	100%	CAD40510	10.1 / 5.12	Yes	TC125017
		TELTFKVAEGSSATS	100%	CAD40509	10.1 / 5.13	Yes	TC129660

^{a)} The observed molecular mass and pl of the protein spots.

^{b)} The predicted molecular mass and isoelectric point of the matched peptides calculated from the ORFs after excluding the predicted signal peptide sequences.


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***:*****:***:*****:*****:*****:*****:*****:*****:*****
Orys2-A: MASLSSFRLSVAVAALLVVGSCATEVTFKVGEGSSGKSLELVTNVAISEVEIKEKGKDW 60
Orys2-B: MASLSSFRLAVAVAALLVVGSCATELTFKVAEGSSASSLELVTNVAISEVEIKEKGKDW 60
Orys2-C: MASMSSFRLAVAAAALLVIGSCATELTFKVAEGSSATSLELVTNVAISEVEVKEKGKDW 60

*.:*****.:***.:*****:*****:*****:*****:*****:*****:*****:
Orys2-A: VALKESSTNTWSLKSEAALKGPFSVRFLVKNGGYRVVDDVIPESFTAGSEYKSGINV 117
Orys2-B: VALKESSNTWTIKSEAPLKGPFSVRFLVKNGGYRVVDDVIPESFTAGSEYKSGIQL 117
Orys2-C: VGLKESGSNTWTLKSEAPLKGPFSVRFLVKNGGYRVVDDVIPESFTAGSEYKSGIQL 117

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Figure 5.3. Multiple alignment of the deduced amino acid sequences of three isoforms of Ory s 2 protein. The deduced amino acid sequences of Ory s 2-A (TC114263), Ory s 2-B (TC125017) and Ory s 2-C (TC129660) are aligned using ClustalX program (Thompson). The "*" symbol indicates that the residues in that column are identical in all sequences in the alignment and the ":" 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 in red. The blue bar on the top of the columns shows the location of the pollen allergen domain (PFAM accession PF01357) in the alignment.

an expansin cellulose-binding domain (EXPANSIN_CBD, PROSITE accession PS50843), which matches to amino acid residues 47 to 113. Both of the domains are the characteristic of group I and group II pollen allergens.

Figure 5.4 demonstrates the alignment of the deduced protein sequences with amino acid sequences of known group II pollen allergens in the database. The Ory s 2 isoforms share comparable amino acid sequence identities (41.7% to 43.5%) with group II pollen allergen proteins of orchard grass, Bermuda grass, timothy grass, wheat, velvet grass and Kentucky blue grass. Phylogenetic analysis, which was based on the multiple sequence alignment, yielded a phylogenetic tree as shown in Figure 5.5. On this phylogenetic tree, the isoforms of Ory s 2 form a distinct subgroup, well separated from the other group II pollen allergens. Sequence alignment analysis also indicated 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 to 267) of rice group I pollen allergen (Ory s 1), respectively (sequence alignment not shown).

5.3.4 Immunoblot analysis

Immunological approaches were employed to see if there was any significant immunological cross-reactivity among the two identified protein spots. Western immunoblot analyses of 2-DE separated pollen proteins (in pH range of 4 to 7) were carried out using the polyclonal antibodies produced against the protein spots Ory s 2-A and Ory s 2-B/C. As expected, the Ory s 2-A antibody detected both protein spots t1-76 and t1-17 (Figure 5.6). However, the Ory s 2-A antibody also cross-reacted to a neighbouring low molecular weight protein spot which was located just below the t1-76, indicating the contamination of the t1-76 protein material used for the injection. A very low detection signal was also present in the gel region corresponding to the rice group I pollen allergens (β -expansins). The antibody produced against Ory s 2-B/C detected both protein spots t1-17 and t1-76, but it did not cross-react with the low molecular weight protein next to t1-76 (Figure 5.7). The control experiments using the pre-immune serum did not detect the same spots in the replica blot. The Ory s 2-B/C antibody was used for the subsequent immunoblot and tissue localisation experiments.

5.3.5 Stage- and tissue-specific expression of Ory s 2

Expressions of Ory s 2 during different pollen developmental stages and in different floral tissues were studied by Western blot immuno-detection analysis. Western blots of rice proteins extracted from rice anthers at four different developmental stages and from different parts of the rice plant, were probed with the anti Ory s 2-B/C polyclonal antibody (Figure 5.8). The results showed that Ory s 2-B/C was not detected in anthers at the uninucleate microspore stage. Reactivity was first detected at a low level in anthers of early binucleate microspore stage and the expression gradually increased to a high level in anthers at the mature pollen stage. A similar level of expression was observed in anther samples of mature pollen stage and pollen that was isolated from the panicle of the same stage. The same immunoblot analysis also revealed that the Orys 2-B/C is only expressed in mature pollen and late stage anther but not in any other part of inflorescence, leaves, shoots or roots of rice.

5.3.6 Cross-reactivity of Ory s 2 to other grasses

The alignment of deduced amino acid sequences of Ory s 2 to group II pollen allergens of other grasses indicated that they share considerable sequence identity. The anti Ory s 2-B/C polyclonal antibody was used to investigate whether there was 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 (Figure 5.9). 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 as demonstrated by our sequence alignment analysis, no cross-reactivity of Ory s 2 to these allergens was detected.

5.3.7 Immunolocalization 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 localization of Ory s 2-B/C in cross sections of rice anthers was performed using immuno-light microscopy. The Orys 2-B/C is exclusively localized to the cytoplasm

Figure. 5.4 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: 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 "*" symbol indicates that the residues in that column are identical in all sequences in the alignment and the ":" and "." indicate that the residues in that column are conserved and semi-conserved, respectively.

	*** **	*:::*	* *	*:	*..::***	*::*	*:::	::****::*	
Orys2-A	--MASLSS-FRLSVAVAALLVVGS-CATEVTFKVGEGSSGKSLELVTNVA--	ISEVEIKEK	55						
Orys2-B	--MASLSS-FRLAVAVAALLVVGS-CATELTFKVAEGSSASSLELVTNVA--	ISEVEIKEK	55						
Orys2-C	--MASMSS-FRLAVAAAALLVIGS-CATELTFKVAEGSSATSLELVTNVA--	ISEVEVKEK	55						
Hol	MSMASSSSSSSLLAMAVLAALFAGAWCPVKVTFTFVEKGSNEKHLAVLVKYEGDTMAEVELREH		62						
LolpII	MSMASSSSSSSLLAMAVLAALFAGAWCPVKVTFTFVEKGSNEKHLAVLVKYEGDTMAEVELREH		62						
Poap2	MSMASSSSSSSLLAMAVLAALFAGAWCPVKVTFTFVEKGSNEKHLAVLVKYEGDTMAEVELREH		62						
Tri2	MSMASSSSSSSLLAMAVLAALFAGAWCPVKVTFTFVEKGSNEKHLAVLVKYEGDTMAEVELREH		62						
PhlpII	MSMASSSSSSSLLAMAVLAALFAGAWCPVKVTFTFVEKGSNEKHLAVLVKYEGDTMAEVELREH		62						
Dacg2	MSMASSSSSGLLAMAVLAALFAGAWCPVKVTFTFVEKGSNEKHLAVLVKYEGDTMAEVELREH		62						
Cynd2	MSMASSSSSGLLAMAVLAALFAGAWCPVKVTFTFVEKGSNEKHLAVLVKYEGDTMAEVELREH		62						

Figure 5.5. **A-** A phylogenetic tree representing the alignment of Ory s 2 proteins with group 2 pollen allergens from other common grasses. The phylogenetic tree was constructed based on the distance matrix between sequence pairs using the MegAlign program of the DNASTAR software package (DNASTAR, Inc, Madison, USA). The scale at the bottom represents the substitution events between the sequence pairs. **B-** The taxonomic classification of 20 common allergenic grass genera according to Andersson *et al* (2003).

A

B

Figure 5.6. 2-DE immunoblot analysis using anti Ory s 2-A serum. 500 µg of pollen protein extract was separated using a 11 cm IPG strip (pH 4-7) in the first dimension and SDS-PAGE in the second dimension. **A.** The separated proteins were electroblotted onto PVDF membrane and probed with anti Ory s 2-B/C serum using ECL-Plus western blot detection kit. The detection signals marked as Ory s 2-A and Ory s 2-B, C are corresponding to the protein spots t1-76 and t1-17 respectively in Figure 5.6-B. **B.** A replica gel of Figure 5.6-A was stained using colloidal coomassie and the protein spots corresponding to the detection signals in Figure 5.6-A are marked.

A

B

Figure 5.7. 2-DE immunoblot analysis using anti Ory s 2-B/C serum. 500 µg of pollen protein extract 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 using ECL-Plus western blot detection kit. The detection signals marked as Ory s 2-A and Ory s 2-B/C are corresponding to the protein spots t1-76 and t1-17 in Figure 5.7-B respectively. **B.** A replica gel of Figure 5.7-A was stained using colloidal coomassie stain and the protein spots corresponding to the detection signals in Figure 5.7-A are marked.

A

B

Figure 5.8. 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 days before heading (DBH); lane 2, anthers at 5DBH; lane 3, anthers at 3DBH 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 using pre-immune serum as a negative control.

Figure 5.9. Cross-reactivity of Ory s 2-A 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 ECL-Plus detection kit. Lane C, heading stage pollen protein was probed using pre-immune serum as a negative control.

and cell wall of mature pollen (Figure 5.10), but no color reaction product was detected in the nuclei region of pollen and anther wall tissues. In control experiments using the pre-immune serum, the anther sections showed a low background color.

5.3.8 Immunoprecipitation

In order to examine if the Ory s 2 proteins interact with proteins of style origin in the rice flower, an attempt was made to co-immunoprecipitate Ory s 2 and other proteins (both anther and style origin) which can form a protein complex with Ory s 2 under native conditions. Figure 5.11 shows a coomassie stained gel of the immunoprecipitate of soluble protein fractions from the anthers at the heading stage and from the anthers, including style tissues, at the late heading stage using the Ory s 2-B/C antibody. Comparison of the protein profiles of immunoprecipitates of anthers and the anthers including styles did not reveal any difference. A band with a molecular weight of 11 kDa, which is corresponding to the Ory s 2 proteins, was present in both the immunoprecipitates that were performed with the Ory s 2-B/C antibody but not in the control experiments which were performed with pre-immune serum. As shown in Figure 5.11, there are a number of high molecular weight protein bands present in increased amount in the immunoprecipitates compared to the control experiment. But this result was not reproducible in a repeated experiment under the more stringent immunoprecipitation conditions.

5.4 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 databases have made it easier to identify these allergens using proteome analysis techniques without using immunological screening and cloning procedures. As described in Chapter 4, during our proteome analysis of rice male gametophyte development, the peptide mass fingerprinting and *N*-terminal amino acid sequence

profiles of two late stage specific proteins were matched to the translation products of three unannotated EST sequences in public databases. Further investigations were carried out using immunochemical and bioinformatic research tools to further characterize the proteins.

Based on 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 analyses of matching TC sequences 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 sequences of the matching ESTs have 23 amino acid long signal peptide sequences. Furthermore, these signal peptide sequences share around 45% of sequence identities 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 it was 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.* 1995a). The evidence presented above 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

Figure 5.10. Immunolight 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-A 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.** The anther cross sections were treated with pre-immune serum and detected in parallel with **A.** (Bars = 100 μ m)

Figure 5.11. Immunoprecipitation of anther and style proteins using anti Ory s 2-B/C antibody. Proteins were extracted under native conditions from anthers plus styles (lane 2 and lane 3) and from anthers alone (lane 4 and lane 5) at the heading stage. Immunoprecipitation experiments were carried out using anti Ory s 2-B/C serum (lane 2 and lane 4). The control experiments were also performed in parallel using the pre-immune serum (lane 3 and lane 5). The precipitated proteins were separated using Novex precast 10-20% Tricine gels and visualised by colloidal coomassie staining. Protein standards were co-migrated in lane 1. Arrows indicate the heavy and light chains of IgG in the immunoprecipitant. A band corresponding to Ory s 2 is also indicated by an arrow.

international allergen nomenclature system (King *et al.* 1994). The *N*-terminal amino acid sequence of Ory s 2-A protein was submitted to the SWISS-PROT protein database with accession number P83466, and the submission of *N*-terminal sequences of Ory s 2-B and Ory s 2-C is pending. The unavailability of serum from patients who are allergic to rice pollen prevented further investigations into the allergenic properties of this group of protein using immunochemical techniques.

Early studies show that grass pollen allergens that belong to the same allergen group but from different grass origins have immunological cross-reactivity (Smith *et al.* 1994a; Smith *et al.* 1994b). Furthermore, cross-reactivity of Ory s 1 with group I allergens from timothy, rye and orchard grasses has been demonstrated (Xu *et al.* 1995b). 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 the anti-Ory s 2 antibody to group II allergens of other common grasses. However, our immunoblot analysis showed that polyclonal the anti-Ory s 2 antibody of rice plant, which is a member of grass subfamily Bambusoideae, does not cross-bind to the counterparts from other three 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 the allergenic epitopes of group II allergens are probably not very conserved among the grass subfamilies. The close conformation between the phylogenetic relationship among the group II allergens and the taxonomic classification of 20 common grass genera also supports this explanation (Figure 5.5). 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 of among the group I allergens can be expected. However, the Ory s 2 antibody cross bound to a ~32 kDa protein band in maize pollen extracts. These proteins presumably are the group I allergens of maize and they have some conserved cross-reactive epitopes with Ory s 2. Zea mI, the group-I pollen allergens of maize, have been identified and characterized using molecular and immunochemical tools and displayed cross-reactivity to group I allergens from other grasses (Broadwater *et al.* 1993). The cross-reactivity between Ory s 2 and other grass

group II pollen allergens needs to be further studied using IgE from rice pollen allergic patients. 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 not very well understood. In terms of biological function, the group I allergens are the best-studied group among the eleven groups of grass pollen allergens. Cosgrove *et al.* reported that group I pollen allergens share low level of sequence homology with expansins and they also demonstrated *in-vitro* cell expansion activities in grass cell walls. Therefore the group I pollen allergens were classified as 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 (Grobe *et al.* 1999; Grobe, Poppelmann *et al.* 2002; Li and Cosgrove 2001). The considerable level 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. Until now, there is no report in scientific journals about the physiological function of group II pollen allergens.

The developmental stage specific expressions and the high abundance of Ory s 2 proteins in mature pollen revealed by our investigation indicates that these proteins should have important functions in pollen development and pollen stigma interactions. In order to gain some knowledge about the biological function of group II allergens, attempts was made to immunoprecipitate and subsequently identify proteins of pollen and style tissue origins which bind to Ory s 2 in native biological conditions. No conclusive differences were observed between the SDS-PAGE profiles of test and control experiments. Optimization of the immunoprecipitation conditions through more repeated experiments was not pursued further because of the limited availability of rice anthers and styles.

Elucidation of the exact biological function of group II allergens using immunochemical and biochemical assays and molecular biology tools in the future will contribute to the better understanding of molecular mechanisms underlying the male

gametophyte developmental processes and also to the elucidation of the precise biological functions of other grass pollen allergens in plant development.

CHAPTER 6 GENERAL DISCUSSION

6.1 INTRODUCTION

In the post-genome sequencing era, proteomics has become an important functional genomics tool providing biological information at the protein level, and has been widely applied to different aspects of the life sciences. The ability of proteomics to deal with large-scale determination of gene and cellular function at the protein level means that it can be used to comprehensively characterize the complex molecular networks underlying various biological processes. This study was directed at investigating the usefulness of 2-DE-based comparative proteomics to study the changes in global protein expression during the male gametophyte development in rice plants, and demonstrates how the potential of this technique can be realized when applied to real biological problems.

6.2 THE ACHIEVEMENTS OF THIS THESIS

Chapter 3:

- The male gametophyte developmental process in rice cultivar Doongara was studied to identify unique cytological “markers” for the major cellular events which take place inside the developing anther.
 - The allometric relationships between different cytological events and vegetative growth measurements such as anther length, auricle distance and the days before heading were used to define discrete developmental stages in male gametophyte development in rice cultivar Doongara. This enabled the collection of near homogenous anther populations for each developmental stage.

Chapter 4:

- Anther proteome maps were established within the pH ranges of 4-7 and 6-11 for six microspore developmental stages. More than 3,500 protein spots were detected by silver staining in the combined pH range of 4-11.
- Comparison of the proteome maps revealed that the expression levels of 150 protein spots changed at different stages during the development. Putative identities

were assigned to 49 protein spots by peptide mass fingerprinting analysis. A further eight low molecular weight protein spots were identified by N-terminal sequencing.

- The integration of the protein expression and identification data demonstrated the active participation of three different signaling and metabolic pathways in the later stages of pollen development.

Chapter 5:

- Three isoforms of developmentally expressed rice homologues of grass group II pollen allergens (Ory s 2) were identified and characterized using bioinformatics. The N-terminal sequence of one isoform has been deposited in the public protein database
- Polyclonal antibodies were produced against Ory s 2 proteins using gel-separated and nitrocellulose-bound proteins as the antigen. The antibodies were used for the further characterization of these proteins.
- Rice group 2 pollen allergens displayed low levels of sequence similarity and cross reactivity with their counterparts from other common grasses.

6.3 RICE AS A MODEL SYSTEM FOR MALE GAMETOPHYTE RESEARCH

One of the achievements of this thesis is its contribution to the use of rice plants as a model system to study the male gametophyte development in crops. As outlined in section 1.2.4.3, several features of rice plants make them an ideal model system for plant research (Goff 1999; Izawa and Shimamoto 1996; Salse *et al.* 2002). These include the availability of the complete draft rice genome sequence and the large number of rice EST sequences, both of which are critical to the use of peptide mass fingerprinting for high-throughput identification of proteins from 2-DE gels. The only impediment to the use of rice as a model for the study of male gametophyte development is the relative difficulty of collecting sufficient amounts of homogenous anther materials at the different developmental stages. This bottleneck is caused by the small size of rice anthers and the heterogeneous maturation pattern of rice flowers on the rice panicle. The cold susceptible Australian rice cultivar Doongara was chosen for this project with the expectation that the experimental results of this project can be

integrated with other ongoing research projects of our group investigating the cold-induced male sterility in rice using the same cultivar. As presented in Chapter 3, the extensive cytological and growth analyses were conducted in this cultivar to define the allometric relationship between growth measurement and microspore developmental stages. These studies provided the basis for the selective collection of a homogenous anther population for each predefined microspore developmental stage. In combination with continuous plant growth throughout the year under a controlled environmental facility, the micro dissection of rice spikelets provided sufficient anther materials for proteomic profiling of male gametophyte development.

The research on this model system resulted in the identification of new male gametophyte-specific genes and also in the detection of molecular networks which are active in the developing rice anthers. Further exploitation of this approach promises to generate further insight into plant reproductive development.

6.4 PROTEOMICS AS A RESEARCH TOOL TO STUDY MALE GAMETOPHYTE DEVELOPMENTAL PROCESS

In the post genomic era, proteomics has emerged as a valuable analytical tool to deal with the analysis of whole genome at the protein level. The unparalleled capacity of 2-DE to simultaneously display thousands of proteins has been utilized in plant research to differentially display the protein contents of various plant tissues under particular physiological conditions, such as biotic and abiotic stresses and plant microbe interactions (Imin, Kerim *et al.* 2001; Natera, Guerreiro *et al.* 2000; Rossignol 2001; van Wijk 2001). In order to expand the application of proteomics in plant science, this thesis set out to systematically investigate one of the fundamental reproductive developmental processes in plants using 2-DE based comparative proteomics. The proteome maps of six developmental stages of anthers were established, new proteins were identified and some of the metabolic and signaling pathways involved in rice anther development were detected. Overall, the primary findings of this thesis demonstrated the potential of applying proteomics to other aspects of plant developmental biology.

However, it also became apparent that the current 2-DE based proteomics technology is not free from problems. Protein solubility and the ability to detect low

abundance proteins are major limitations, which results in the incomplete representation of proteomes on 2-DE gels. Hydrophobic proteins, which represents up to 30% of the genome coding capacity of eukaryotic cells (Wallin and von Heijne 1998), are generally underrepresented in proteome maps due to their low solubility in currently available detergents which are compatible with 2-DE. The total number of predicted genes from draft sequences of *japonica* rice genome range from 32,000 to 61,000 (Delseny 2003; Goff, Ricke *et al.* 2002). In this study, the 3,500 rice anther proteins displayed on 2-DE gels only accounts for 10% of the minimum coding capacity of the rice genome, assuming one gene product per estimated gene. This figure will decrease further if the number of protein isoforms detected in this study is taken into the account. Furthermore, the transcriptional analyses of pollen gene expression in monocot plants have estimated that about 20,000 and 24,000 different mRNAs are present in the mature pollen grains of *Tradescantia* and maize, respectively (Mascarenhas 1990). If it is assumed that the similar numbers of genes are expressed in rice pollen, the proteins detected on the anther 2-DE maps could not fully reflect the true complexity of eukaryotic gene expression.

Another major limitation lies in the area of post-separation protein identification techniques. As shown in Chapter 4, the success rate with protein identification by PMF analysis was lower than expected in this study. The protein identification by *N*-terminal analysis and peptide mass fingerprinting of eight protein spots using the same database resulted in the totally different success rates. The eight protein spots, which had not been identified by PMF analysis, were successfully identified by *N*-terminal sequencing analysis. This indicated that the under representation of the matching database entries was not the main reason contributing to the failure of protein identification of these eight protein spots by PMF analysis. Furthermore, the searches of different databases using the same PMF data also resulted in different success rates (section 4.3.3). These examples highlighted the imperfectness of PMF analysis and the importance of database quality in the success rate of protein identification. The real capacity of proteomics can be fully utilized if only the complete or near complete proteome of a tissue or cell is successfully displayed and every protein is identified.

The current experimental limitations in 2-DE based proteomics can be reduced in a number of ways. Solubilisation of hydrophobic proteins can be improved by the application of new zwitterionic detergents (Chevallet *et al.* 1998) and by sequential

extraction of the sample with reagents of increasing solubilizing power (Molloy *et al.* 1999). Detection and identification of highly hydrophobic proteins can also be achieved by MudPIT technology which based on chromatographic separation of peptides generated from a complex protein mixture (Koller *et al.* 2002) One dimensional SDS PAGE, which is compatible with solubilisation of membrane proteins using SDS, can also used to the separation of highly hydrophobic proteins, even though the resolution of this technique is much lower than 2-DE analysis (Andon *et al.* 2002) Separation of protein samples using narrow range (1 pH unit) IPG strips to generate overlapping proteome contig maps (Cordwell *et al.* 2000) and the enrichment of low-abundance proteins by preparative isoelectric pre-fractionation step using a multi-compartment electrolyser (MCE) can contribute to the visualization of low-abundance proteins (Herbert *et al.* 2001).

6.5 GLOBAL CHANGES IN PROTEIN EXPRESSION DURING THE RICE MALE GAMETOPHYTE DEVELOPMENT

The establishment of 2-DE based proteomics as a viable differential display tool has enabled the investigation of global changes in gene expression of a biological system at the protein level. As presented in Chapter 4, while the global expression pattern of rice anther proteins was largely unaltered, the comparisons of rice anther proteome maps showed that the expression level of 150 proteins changed more than two fold during the course of anther development. These 150 proteins account for less than 5% of the total proteins that were visualized on 2-DE gels. Most significant changes in protein expression levels were detected between the proteome maps of the pollen mother cell stage and the heading stage. More than 50 new protein spots were newly expressed at the heading stage compared to the pollen mother cell stage.

Transcriptional profiling studies of male gametophyte development showed that in pollen grains of *Tradescantia* and maize, the male gametophyte specific genes could be classified into “early” and “late” genes (Mascarenhas 1990). According to those studies, most of the “early” genes were transcriptionally active just after pollen meiosis. The “late” genes become active soon after first pollen mitosis and are thought to play major roles during the later part of pollen maturation and during pollen germination. The transcripts of some “late” genes are stored untranslated in pollen grains until pollen germination (Stinson, Eissenberg *et al.* 1987). The late stage-specific proteins that were

Figure 6.1. Temporal expression patterns of some differentially displayed protein identified in this study.

detected in this study are presumably the products of the “late” genes, because the expression of most of these proteins starts at around the first pollen mitosis (Table 4.1, Chapter 4), which conforms to the above-mentioned temporal expression pattern of “late” genes. Furthermore, the comparison of proteome maps also revealed a small number of proteins representing the “early” class gene products. However, the number of the detected early stage-specific proteins is much less than those of late stage-specific proteins in this study. A possible explanation for this is that the abundance level of “early” gene products is probably below the limit of detection of the silver staining used in this study. It is also possible that some transcripts of the “early” genes remain untranslated until the onset of certain later stages. The examples of “early” and “late” gene products, which were identified in this study, are shown in Figure 6.1. These results of this study clearly demonstrate the potential power of proteomics to test and complement the transcriptional profiling studies at the protein level.

6.6 METABOLIC AND REGULATORY PATHWAYS INVOLVED IN POLLEN DEVELOPMENT

Out of the 150 differentially displayed protein spots, 49 proteins were tentatively identified by PMF analysis and eight proteins by *N*-terminal sequencing and homology searches. In order to better understand the molecular networks in which these proteins are involved, putative functional classifications were assigned to the identified proteins according to the functional classifications in *A. thaliana* and *M. truncatula* proteome projects (Kaul, Koo *et al.* 2000; Mathesius, Keijzers *et al.* 2001). A statistical summary of the functional classification is shown Figure 6.1. Largest group of proteins (26%) are involved in metabolism and the next largest group matched to hypothetical or unknown proteins. Unlike other studies, the proteins involved in cell wall and cytoskeleton formation constituted the third largest group (18%), perhaps indicating the importance of these processes in pollen development. Several functional protein classes such as cell signaling and protein synthesis that have been identified in other projects are underrepresented, probably because of the low number of identified proteins in this study.

Figure 6.2. Summary of the functional classification of the identified rice anther proteins.

Based on the predicted identities of the limited number of differentially displayed anther proteins and their temporal regulation patterns, three developmentally regulated metabolic or regulatory pathways were identified that are active in developing rice anthers. Table 6.1 lists the predicted identities of three groups of proteins and their proposed biological functions in cellular processes in which they are involved. The ubiquitin/26 proteasome pathway, of which three components were identified, is involved in the selective proteolysis of proteins in plants (discussed in Chapter 4). Recent analysis of *Arabidopsis thaliana* genome revealed that a sizable fraction (~5%) of the plant proteome is devoted to encoding the components of this pathway and the individual components are connected to almost all aspects of plant biology (Fu *et al.* 1999; Vierstra 2003). The up-regulation of two components indicates the possible role of COP9 signalosome-mediated light signal sensing in anther dehiscence at the flowering stage. The up-regulation of three proteins involved in starch and sucrose metabolism at later stages of pollen development coincides with the increased starch synthesis activity in pollen grains. Late stage-specific expression of B-expansin, profilin and actin conforms to their proposed function in cell wall expansion and pollen stigma interaction.

Considerable portion of the identified proteins in this study are primary metabolic enzymes which are involved in basic “housekeeping” metabolic processes in plants. However, it is possible that these housekeeping proteins have other function(s) during

Table 6.1. Summary of the proteins involved in metabolic pathways

Pathway	Identity	Isoform number	Proposed Function
Ubiquitin / 26 proteasome	Jab 1/CSN5	1	repression of photomorphogenesis
	Ubiquitin	1	protein degradation
	20S	1	protein degradation
	proteasome		
Starch /sucrose metabolism	Vacuolar acid invertase	2	hydrolysis of sucrose
	Fructokinase II	2	fructose phosphorylation
	Beta-galactosidase	2	hydrolysis of O-glycosides
Cell wall and cytoskeleton	Actin	1	cytoskeleton formation
	Profilin	3	actin binding
	Beta-expansin	3	cell wall loosening
	Group II allergens	3	cell wall loosening ??

the male gametophyte development in anthers in addition to their main role in primary metabolism. Recent studies showed that some proteins could “moonlight”, or have more than one distinct function in an organism. Therefore, they are called moonlighting proteins (Jeffery 1999). The function of moonlighting proteins can vary as a consequence of changes in cellular localization, cell type, oligomeric state, or the concentration of a ligand, substrate, cofactor or product. One of the identified proteins in this study, quinone oxidoreductase, has been reported to have moonlight functions in animals (Piatigorsky 1998). Recent results by the Johnston *et al.* show that the regulatory proteins of 26S proteasome complex, of which a subunit was identified in this study, has a role in nucleotide excision repair and transcription elongation (Gonzalez *et al.* 2002; Jeffery 2003). This moonlighting mechanism of proteins adds another dimension to the cellular complexity of eukaryotic gene expression.

Regarding the significant cellular changes that take place inside anthers during the development, the differential protein expression profiles that were obtained in this study are probably an under representation of protein changes in developing anthers. Therefore, it is still inadequate to provide a full insight into the molecular mechanism of male gametophyte developmental process. Complete achievement of such a goal in this study was impeded mainly by the current limitations in 2-DE and protein identification techniques (discussed in section 1.3.5).

6.7 FUNCTION OF IDENTIFIED PROTEINS IN COLD INDUCED MALE STERILITY

One of the aims of this study is to assist the research projects aimed at understanding the molecular mechanism of cold-induced male sterility in rice (abnormal development) by providing information about proteins involved in the normal development of rice anthers. Two of the identified proteins in this study, β -expansin and nucleoside diphosphate kinase, have been found to be involved in cold-induced male sterility in rice anthers by proteomic analysis (Imin 2002). An increase in the partial degradation or cleavage of these proteins was detected in trinucleate anthers which had been treated with cold shock at the young microspore stage. The involvement of β -expansin in the process of cold-induced male sterility suggests that the sterility resulting from cold treatment is possibly partially attributed to incomplete pollen tube growth. In

the same study, the β -6-subunit of 20S proteasome was identified and observed to be up-regulated by two fold in response to cold treatment at the young microspore stage. The identification of another subunit of the 20S proteasome and other constituents of ubiquitin-proteasome pathway in our study provided further information about the cellular processes in which this cold responsive protein (β -6-subunit of 20S proteasome) is involved (section 4.4.1). Future studies in cold-induced male sterility can be directed at the stage-specific anther proteins and the relevant biological pathways identified in this project. This will contribute to the understanding of molecular mechanism of the stress-response process in plants.

6.8 BIOLOGICAL FUNCTION OF POLLEN ALLERGENS IN PLANT REPRODUCTION

Grass pollen allergens have been studied extensively since the 1960s, and to date, eleven different groups of grass pollen allergens have been identified and characterised from one or more species (Andersson and Lidholm 2003; Suphioglu 2000). The pollen allergens have been poorly characterized in terms of their biological function in plants, although their immunological features have been well studied. Out of the eleven groups of pollen allergens characterised in grasses, biological functions have been proposed for only a few groups based on the sequence homology to other known proteins or based on the results of *in vitro* biochemical assays (Andersson and Lidholm 2003). The group I allergens are thought to have an expansin activity and to play important roles in pollen tube growth during fertilization (Cosgrove 1997; Cosgrove, Bedinger *et al.* 1997). The group 5 allergen of timothy grass pollen has been proposed to have ribonuclease activity (Bufe *et al.* 1995). The profilins are involved in signalling pathways that regulate the pollen cytoskeleton remodelling during the pollen engorgement and pollen tube growth (Vidali and Hepler 2001).

In this study, multiple isoforms of three groups of rice pollen allergens have been identified. These allergens included three isoforms of group I allergen (β -expansin), three isoforms of group 12 (profilin) and three isoforms of group II allergens. In mature pollen, these three groups of allergens are the most abundant proteins. The comparison of proteome maps from different developmental stages demonstrated a very similar temporal expression pattern of these allergens during the anther development. The

expression of β -expansin and profilin was first detected at the early or late binucleate stages and steadily increased to high levels at the pollen maturity. The expression of these proteins coincides with the formation of exine and intine wall layers and enlargement of pollen grains, indicating the involvement of these proteins in pollen cell wall structure and cell enlargement (discussed in Chapter 4).

Multiple isoforms of group II pollen allergens were identified in this study using a combination of proteomics and bioinformatics. As revealed by immunoblot analysis, their expression is specific to pollen, and they also displayed a temporal regulation pattern which is similar to β -expansins, implying their possible involvement in pollen development and fertilization processes. Until very recently, there was no report in scientific journals about the physiological function of group II pollen allergens. Li *et al.* very recently reported that the group 2 pollen allergens from ryegrass and timothy grass exhibited plant cell wall-loosening activity which is similar to β -expansins (Li *et al.* 2003). They concluded that at least part of the characteristic cell wall-loosening action of β -expansin is attributable to its C-terminal domain, which shares a high level of sequence homology with the corresponding group 2 allergens. Even if the cell expansion activity of the group II pollen allergens is confirmed, further research still needs to be done before the biological significance and exact molecular mechanism of redundant cell expansion activities of the group I and group II pollen allergens in plant reproductive development are elucidated.

6.9 PRODUCTION OF POLYCLONAL ANTIBODIES USING A COMBINATION OF 2-DE AND NITROCELLULOSE BOUND ANTIGEN

During the past few decades, immunocytochemical methods have become powerful and indispensable investigation tools for different aspects of plant science. The application of immunochemical methods in plant research often involves the production of monoclonal and polyclonal antibodies directed against some minor proteins, or against nonsoluble or membrane proteins, which are difficult to purify in native form by liquid-based fractionation approaches. In addition to its role as a differential display tool, the 2-DE has proved to be a quick and efficient method of obtaining pure antigenic material for immunization from crude or partially purified protein extracts (Diano and

Bevic 1997). Once proteins are separated on 2-DE gels, the protein spots of interest can be excised from the lightly stained gels, ground and injected into animals with or without adjuvant. The generally poor results derived from this approach may be due to the toxicity of SDS and acrylamide in the antigen which cause intensive tissue and cell damage, resulting in the early death of the experimental animals (personal communication with Dr Nijat Imin, Australian National University, Canberra). As an alternative approach, the 2-DE separated proteins can be transferred to nitrocellulose membrane and the protein-bearing nitrocellulose is then solubilized by physical or chemical means before being administered to animals (Knudsen 1985). As demonstrated in Chapter 5, the antibody production to the 2-DE-purified Ory s 2 proteins was successful by this method using as little as 50 µg proteins. The antibody production elicited to the low molecular weight proteins, without conjugation to carrier proteins, was probably partially attributable to the intrinsic allergenic nature of the Ory s 2 proteins. Another factor that contributed to the good immune response might be the adjuvant role of the nitrocellulose acting to slowly release the bound antigens, and thereby elicit a good immune response in animals.

The antibody production to 2-DE separated proteins in this study has demonstrated the parallel application of 2-DE based proteomics as a micropreparative tool in addition to its main application as a large-scale analytical approach. However, it should be kept in mind that the injection of a distinct protein spot does not necessarily result in the production of a mono-specific antibody as one protein spot on a gel can contain more than one protein population because some proteins with the similar *pI* and molecular weight can co-migrate to the same gel positions. As presented in Chapter 4 (Table 4.2), this is the case in two out of eight protein spots (25%) which were subjected to *N*-terminal sequencing. Therefore, special care should be taken while producing antibodies to 2-DE proteins identified by PMF analysis, because it is very difficult to confirm the homogeneity of the analyzed spots from the generated PMF data. Application of narrow range IPG strips in the micro- preparative 2-DE should increase the chance of obtaining homogenous antigens for antibody production.

6.10 CONCLUSION AND FUTURE PERSPECTIVES

This thesis has demonstrated the usefulness of 2-DE based proteomics for investigating the changing pattern of gene expression at the protein level during

developmental processes in plants. In order to successfully follow a developmental sequence it is vital to have access to homogenous cell populations representing particular developmental stages. Otherwise, the real changes in protein expression levels will remain diluted or swamped by the overlapping or more abundant protein population. In this project, a considerable effort was made collect near homogenous anther populations by excluding the non-anther parts of the rice flower. This effort certainly resulted in the partial enrichment of some anther-specific and low abundant proteins.

An alternative method to collecting homogenous anther samples from rice flowers at different developmental stages is to subject the mixed microspore population to fractionation in a percoll or sucrose gradient (Fan, Armstrong *et al.* 1988). Although highly uniform microspore populations representing different developmental stages can be obtained in sufficient amounts, this method is not suitable for the isolation of pre-meiotic and early young microspores which lack of rigid pollen wall. As presented in Chapter 5, the isolation of mature pollen grains and the analysis of pollen proteins by 2-DE have proven successful in our study. Unfortunately, a similar research approach has not been extended to the study of microspores from other stages due to the time limitation of the PhD project.

The development of methods for the isolation of generative cells, sperm cells, embryo sacs and egg cells from angiosperm plants has opened up new possibilities for plant reproductive research, utilizing proteomic and transcriptional profiling of generative or sperm cells. Viable sperm or generative cells can be isolated from the pollen grains or growing pollen tubes by enzymatic digestion, osmotic bursting or squashing, filtration and centrifugation (Matthysrochon *et al.* 1987; Theunis *et al.* 1991). In several plant species, the isolated sperm and generative cell has been subjected to transcriptional profiling, resulting in the identification of generative cell specific-genes and in the isolation of the male gametic cell-specific promoters (Singh *et al.* 2003; Xu, Swoboda *et al.* 1999b). McCormick's laboratory recently initiated a large-scale project to construct and sequence cDNA libraries from isolated sperms, eggs, and embryo sacs of maize (for details visit <http://www.pgec.usda.gov/McCormick/McCormick/mclab.html>). Similar studies have also been conducted at the protein level in *Plumbago zeylanica* L (Geltz and Russell 1988). The proteins extracted from sperm, cytoplasmic particulate and water-soluble fractions of mature pollen have been

separated and compared. However, so far there is no report about the large-scale profiling of sperm or generative cell proteins in crop plants. Large-scale proteome profiling of isolated generative or sperm cells will provide a deeper insight into the protein composition of male gametophytes.

In parallel to proteomic profiling, it is also important to study the dynamic changes of gene expression during the male gametophyte development at the transcriptome level using large scale and high-throughput mRNA profiling techniques such as SAGE (serial analysis of gene expression) and DNA microarray analysis. This may enable the identification of some genes whose protein products are not displayed by 2-DE analysis. At the same time, the large-scale biochemical profiling approach, which is also known as metabolomics, can be applied to profile the changes of chemical constituents of male gamete cells at different developmental stages. This approach can complement the proteomic and transcriptomic analysis with functional data at the metabolome level.

It is hoped that in the new era of Systems Biology, technological improvements in proteomics and other global expression profiling tools will ultimately lead to the more complete unraveling of the molecular and biochemical mechanisms underlying male gametophyte developmental processes in crop plants. The knowledge derived from such studies can readily be exploited to resolve the practical problems such as cold- induced male sterility and self-incompatibility in plants, and will eventually contribute to the improvements of crop plants to the benefit of ever increasing world population.

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APPENDIX

Refereed Publications

1. **Kerim, T.**, Imin, N., Weinman, J. J. and Rolfe, B. G (2003). Proteome analysis reveals developmentally expressed rice homologues of grass group II pollen allergens, *Functional Plant biology*, 30 (8): 843-852.
2. **Kerim, T.**, Imin, N., Weinman, J. J. and Rolfe, B. G. (2003). Proteome analysis of male gametophyte development in rice anthers, *Proteomics* 3(5): 738-51.
3. Imin, N., **Kerim, T.**, Weinman, J. J. and Rolfe, B. G. (2001). Characterisation of rice anther proteins expressed at the young microspore stage, *Proteomics* 1(9): 1149-61.
4. Mathesius, U., Imin, N., Chen, H., Djordjevic, M. A., Weinman, J. J., Natera, S. H., Morris, A. C., **Kerim, T.**, Paul, S., Menzel, C., Weiller, G. F. and Rolfe, B. G. (2002). Evaluation of proteome reference maps for cross-species identification of proteins by peptide mass fingerprinting, *Proteomics* 2(9): 1288-303.
5. Imin N, **Kerim T**, Weinman JJ, Rolfe BG. Effect of Early Cold Stress on the Maturation of Rice Anthers *Proteomics* (in press).