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Proteome analysis of male gametophyte development in rice anthers

We used proteomic analysis to investigate the changing patterns of protein synthesis during pollen development in anthers from rice plants grown under strictly controlled growth conditions. Cytological analysis and external growth measurements such as anther length, auricle distances and days before flowering were used to determine pollen developmental stages. This allowed the collection of synchronous anther materials representing six discrete pollen developmental stages. Proteins were extracted from the anther samples and separated by two-dimensional gel electrophoresis to produce proteome maps. The anther proteome maps of different developmental stages were compared and 150 protein spots, which were changed consistently during development, were analysed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry to produce peptide mass fingerprint (PMF) data. Database searches using these PMF data revealed the identities of 40 of the protein spots analyzed. These 40 proteins represent 33 unique gene products. Four protein spots that could not be identified by PMF analysis were analysed by *N*-terminal microsequencing. Multiple charge-isoforms of vacuolar acid invertase, fructokinase, β -expansin and profilin were identified. These proteins are closely associated with sugar metabolism, cell elongation and cell expansion, all of which are cell activities that are essential to pollen germination. The existence of multiple isoforms of the same proteins suggests that during the process of pollen development some kind of post-translational modification of these proteins occurs.

Keywords: Microspore development / Peptide mass fingerprinting / Rice (*Oryza sativa* L.) / Two-dimensional gel electrophoresis
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1 Introduction

In higher plants the development of the male gametophyte is a well-programmed and elaborate process that plays a crucial role in plant reproduction (for reviews see [1–5]). Male gametophyte development takes place inside a highly differentiated male gametophytic organ, the stamen. In the vegetative apex of very young flowers, the diploid sporophyte cells of the stamen primordia differentiate into both somatic and sporogenic cells. The somatic cells form the anther wall of the stamen by multiple divisions while the sporogenic cells differentiate into meiocytes, or pollen mother cells (PMCs). Each diploid pollen mother cell forms a tetrad of four haploid microspores by meiotic divisions, which remain enclosed within

a callose wall. After the dissolution of the callose wall by $\beta(1,3)$ -glucanase (callase) which is secreted by the surrounding tapetal cells, the free young microspores are released into the anther locule. Thereafter, the exine and germ pore begins to form on the surface of the free young microspore. The developing young microspore undergoes an asymmetric mitotic division, forming a large vegetative cell and a small generative cell that is enclosed within the vegetative cell. This bicellular structure is called the pollen grain. The generative cell inside the pollen grain forms two identical sperm cells by a round of symmetrical mitotic division. At the same time the inner pollen wall, or intine, is formed and starch granules begin to accumulate inside pollen grains. By this time, the panicle heading and anther dehiscence occur, and mature pollen grains are released.

The whole developmental process of microgametogenesis is controlled by coordinated gene expression in both somatic and gametophytic cells. A temporal and spatial regulation of gene expression has been reported during male gametophyte development in higher plants [6]. A number of research programs focused on the molecular biology of anther differentiation have identified several

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Abbreviations: DAPI, 4', 6-diamidino-2-phenylindole; DBH, days before heading; PMC, pollen mother cell; PMF, peptide mass fingerprint

anther-specific genes which are preferentially expressed in anthers of rice and certain other plants [6–13]. However, the function of these anther-specific gene products and the roles of these proteins on pollen developmental pathways are currently unknown.

Rice (*Oryza sativa* L.) is an important crop, which provides staple food for about half of the world's population [14]. Because of its importance, many anatomical features of both the vegetative and reproductive parts of the rice plant have been extensively studied [3, 15, 16]. The molecular biological analysis of rice has also been the main focus of cereal genomic research because rice is an excellent model system for crop research. It has a relatively small genome (~430 Mb), and shares a high degree of synteny with other cereal crop plants. Rice is also the easiest of the cereal plants to transform [17]. These make rice an ideal system for studying the molecular mechanisms of male gametophyte development of crop plants in spite of the physical limitations imposed by the small size of the rice anthers. Rice is also the first crop plant whose genome has been sequenced. Recently, two groups simultaneously published drafts of rice genome sequence of the two major rice subspecies, *indica* and *japonica* [18, 19]. As the rice genome sequence has become publicly available, the prediction of possible open reading frames (ORFs) by bioinformatics and the understanding of the function of gene products are the first steps of exploiting this wealth of genome sequence information. However, there is not a strict linear relationship between genes and their protein products. The prediction of an ORF in genomic data by sophisticated gene prediction programs does not necessarily imply the existence of a functional gene or the translation of a protein [20]. Therefore, the verification of the existence of a gene product in the protein complement of a living organism is an important step in understanding the function of a gene. In this respect, 2-DE based proteomic analysis is the best complementary research tool to genomics.

The 2-DE based proteomic approach has already been applied to study the differential protein synthesis and protein phosphorylation patterns of *Brassica napus* microspores during microspore embryogenesis [21]. The pollen coat proteins of self-incompatible and self-fertile *Festuca pratensis* were also profiled by applying 2-DE based technology [22]. In the field of rice study the proteomic approach has already been applied to cataloguing the proteome of the different tissues of rice plants [23–27]. Agrawal *et al.* [28] employed a proteomic approach to investigate ozone stress responsive proteins in rice seedlings. Drought and salt stress-responsive leaf and root proteins were also revealed by a comparative prote-

omic approach in rice [29]. Recently, a systematic and most comprehensive proteomic analysis of leaf, root and seed tissues of rice was reported [30]. In this report, Koller and colleagues identified 2528 unique proteins by using 2-DE followed by MS/MS and multidimensional protein identification technology. They identified tissue specific distinct regulatory pathways by a comparative display of proteomes of different rice tissues. Here we report the application of proteome analysis techniques to a plant developmental biology system. We have investigated the temporal changes in protein synthesis and post-translational modifications of some proteins during the male gametophyte development in rice. Identification and characterization of anther-specific genes and their protein products has enabled us to obtain some insight into the possible pathways and protein interaction networks underlying the complex cellular process of pollen development.

2 Materials and methods

2.1 Reagents

The reagents and apparatus used have been previously described in detail [31]. All chemicals are of analytical or electrophoresis grade. Unless stated all the chemicals were purchased from Sigma (St. Louis, MO, USA). MilliQ quality water with resistance of higher than 18 M Ω was used throughout the experiments.

2.2 Plant growth

The Australian rice cultivar, Doongara, was grown under controlled environment conditions as described [23]. Once the plants were established they were reduced to one plant *per* pot. Four weeks after sowing, urea (15 g/m²) was applied as nitrogen fertiliser and the plants were flooded. Just before panicle initiation stage the plants were transferred to a growth chamber maintained at a temperature of 30/20°C (12/12h), 70% relative humidity and a photon flux density of 330 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

2.3 Cytological study

To establish cytological controls, we conducted cytological studies on rice microspore development using acetocarmine and DAPI (4', 6-diamidino-2-phenylindole) staining techniques. A few days before the panicle booting stage the rice plants were dissected and cytological studies were used to predict the correlation between early microspore developmental stages and vegetative growth measurements such as anther length and auricle dis-

tance. First, the auricle distance of each plant were measured and recorded. Then the lengths of the anthers of the top five spikelets of the top three primary branches were measured with a binocular microscope equipped with an ocular micrometer using $2\times$ and $4\times$ objectives. The developmental stage of each anther was subsequently determined by the acetocarmine anther squash technique as previously described [32]. The number of days before heading stage was used as a growth measurement to predict the late stages of microsporogenesis. DAPI staining was used to visualize the pollen nuclei to determine the late microspore developmental stages as described before [33]. DAPI was stored at 4°C as a 1 mg/mL stock solution in distilled water. One microliter of this stock solution was diluted in 1 mL of 0.1 M citrate phosphate buffer (pH 4) containing 1% Triton X-100 and fresh anthers, collected every morning from plants at various days before heading stage, were dissected in this solution to release the microspores. The microspores were incubated in this solution for 30 min under dark conditions and observed under a Nikon Optiphot light microscope using UV filter (main wavelength 365 nm). Photographs were taken on Fujichrome 400 colour slide film using a Nikon FX-35 A camera.

2.4 Anther sampling

The early stages of microsporogenesis were predicted according to auricle distance and the days before heading. The spikelets of top three primary branches were dissected with fine forceps and scalpels under a dissection microscope to remove the enclosed anthers. In order to take more synchronous anther samples only the main tillers of the rice plants were used for the experiments. Anthers within the same anther length range were collected and immediately frozen in liquid nitrogen and stored at -80°C until use. Anther samples belonging to the same anther developmental stages were pooled to obtain 100 mg starting sample for protein extraction. For early stages around 3000 anthers were needed to obtain 100 mg anther material, whereas for late stages around 700 anthers were needed.

2.5 Protein extraction

The anther proteins were extracted as described before using TCA-acetone precipitation method [23, 34]. The protein concentration of the supernatant was determined by Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA) with a series of concentrations of BSA as the standards. Extracted proteins were stored in 100 μg aliquots at -80°C .

2.6 2-DE, gel staining and image analysis

2-DE was done as previously described [23, 31] in a Multiphor II horizontal electrophoresis system (Amersham Biosciences, Uppsala, Sweden). IEF was done using 18 cm or 24 cm, pH 4–7 linear IPG strips (Amersham Biosciences). For silver staining, a 100 μg aliquot of total soluble protein was diluted in sample buffer to a final volume of 100 μL and loaded onto the anodic end of each IPG strip using a sample-loading cup. For preparative gels, 1 mg of soluble protein was loaded in the same way. The second dimension separation was carried out using pre-cast 12–14% ExcelGel SDS gels (Amersham Biosciences) on a Multiphor II horizontal electrophoresis unit. Low molecular weight protein standards (Amersham Biosciences) or Mark12 wide-range protein molecular weight standards (Invitrogen, Leek, The Netherlands) were coelectrophoresed in the second dimension. After the second dimension separation, the proteins on analytical 2-DE gels were visualized by silver staining according to Rabilloud [35] with some minor modifications. As an additional buffer, 2.5 mM hemisodium salt HEPES (Research Organic, Cleveland, OH, USA) was added to the silver nitrate solution. The proteins on preparative gels were stained using the colloidal Coomassie method [34]. The experiments were repeated at least three times for each developmental stage.

The stained gels were scanned at a 600 dots *per* inch resolution with a UMAX Astra-2400S scanner (UMAX Technologies, Fremont, CA, USA). The spot detection and gel comparison were made using MELANIE 3 software (Swiss Institute of Bioinformatics, Geneva, Switzerland). The relative M_r s of the proteins were determined by the comigration of the protein standards using MELANIE 3 software.

2.7 MALDI-TOF MS analysis

Selected protein spots, which were differentially displayed during microspore development, were manually excised from the colloidal Coomassie stained preparative gels for analysis using MALDI-TOF MS to generate PMF profiles. MS analysis was done at the Australian Proteome Analysis Facility at Macquarie University in Sydney with a Micromass ToFSpec 2E time of flight mass spectrometer (Waters, Milford, MA, USA). The protein samples in gel pieces were treated for 16 h of tryptic digestion with 8 μL of 15 ng/ μL sequencing grade modified trypsin (Promega, Madison, WI, USA) at 37°C . The resulting peptides were extracted from the gel piece with 50% v/v acetonitrile, 1% v/v TFA solution. A 1 μL aliquot was spotted onto a sample plate with 1 μL of matrix containing 8 mg/mL α -cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile.

and 1% TFA solution. The spectra were acquired in reflectron mode in the mass range of 600–3500 Da. Two trypsin autodigestion peaks at 842.51 Da and 2211.1 Da were used for internal calibration of each spectra.

2.8 Electroblothing and *N*-terminal sequencing

The proteins resolved on preparative 2-D gels were electroblotted onto PVDF membranes (Bio-Rad) as described elsewhere [31, 36]. Selected spots were excised from the membranes for *N*-terminal amino acid sequencing. The *N*-terminal microsequencing was done on a PROCISE 494–01 sequencer system (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) at the ANU Biomolecular Resource Facility.

2.9 Database searching

The PMF data was used to search against the SWISS-PROT and NCBI non-redundant public protein database using the search engine ProFound available at http://129.85.19.192/profound_bin/WebProFound.exe. The taxonomic category of *O. sativa* was searched first. If no significant match was found, then the whole Viridiplantae (green plants) database was searched. During the searches all the peptide masses were assumed to be monoisotopic and a mass accuracy of 100 ppm was used. A maximum of one missing cleavage site and modification of cysteines to carboxyamidomethyl cysteine (Cys_CAM) by iodoacetamide or to propionamide cysteine (Cys_PAM) by acrylamide monomers were considered during the searches. At the same time, the PMF data were also used to search against the The Institute for Genomic Research (TIGR) Rice Gene Indices database available at <http://www.tigr.org/>. The Tentative Consensus (TC) and EST sequences (OsGI Release 7.0 – May 29, 2001) were downloaded and translated in six reading frames to generate the possible translation products of the EST sequences. The FLIP program developed by the Organella Genome Mega-sequencing Program (OGMP) was used to translate the EST sequences, available at the OGMP website <http://megasun.bch.umontreal.ca/ogmp/ogmpid.html>. Then PMF homology searches of this custom database were done using MassLynx software V 3.4 (Micromass, Waters, Milford, MA, U.S.A.).

The identification of protein spots by *N*-terminal amino acid sequences homology was done in two steps. First, the *N*-terminal amino acid sequences homology search was done with the FASTA program (available at the EMBL web site <http://www2.ebi.ac.uk/fasta3/>) to screen nonredundant protein databases to find possible matches. If no significant matches were found, then

BLAST search was conducted using the theoretical six-frame translation of rice EST sequences stored at the TIGR Rice Gene Indices database. The translated amino acid sequences of the matching candidate ESTs were analyzed for possible signal peptide cleavage sites using the on-line signal peptide prediction program SignalP, [37] available at <http://www.cbs.dtu.dk/services/SignalP/>. The theoretical M_r and p/s of the matching sequences, excluding the signal peptide, were calculated using the on-line Compute pI/Mw tool available at the ExPASy Molecular Biology Server (<http://expasy.org/>). The agreement between the theoretical p/s and molecular masses of the matched sequences and those of experimentally observed was taken into the consideration during PMF and *N*-terminal homology searches.

3 Results

3.1 Prediction of pollen developmental stages

In order to collect enough synchronous anther samples for proteome analysis of each discrete developmental stage, it was essential to establish a reliable, nondestructive growth measurement to enable the assessment of the precise cytological stage of microspore development within the inflorescence. More than 200 plants from three different batches were dissected for cytological analysis in order to investigate the correlation between the developmental stages and vegetative growth measurements, such as the length of anther and the number of days prior to the heading stage. Some results of cytological analysis are shown in Fig. 1. For the practical need of collecting enough anther materials, the whole period of development from pollen mother cell stage to anthesis were divided into seven stages based on the cytological observation and concomitant growth measurements as seen in Table 1. From the results of cytological analysis we found that microspore development during early stages was accompanied by a progressive increase in anther length. We used anther length as the main external growth measurement in combination with auricle distance in assessing the early three stages of microspore development. The tetrad stage is transient thus making it very hard to collect enough anther material for 2-DE analysis. Therefore, we combined pollen mother cell and tetrad stages in anther sampling in our experiments. The anther length cannot be used as a growth measurement for assessing the late microspore stages because the anthers attain their full length soon after the first pollen mitotic division. Instead, the number of days prior to heading was used as a growth measurement. Our experimental data obtained by observing the growth profile of panicles showed that it took 13–14 d from the first reduction division of pollen mother cells

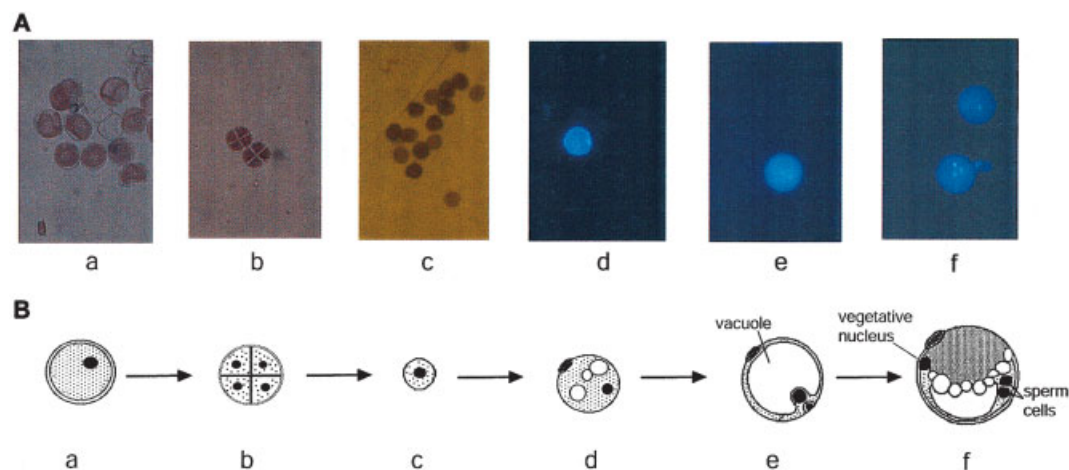


Figure 1. Microspore development and pollen maturation in rice. (A) Micrographs of developing pollen grains at different developmental stages: a-c were stained with acetocarmine and d-f were stained with DAPI. a, pollen mother cell; b, tetrad, c, early young microspore, d, uninucleate microspore, e, binucleate microspore, f, trinucleate pollen grain. (B) Schematic representation of A (modified from Bedinger) [1].

Table 1. The microspore developmental stages in rice cultivar Doongara

Stage of development	Range of anther lengths (μm)	Range of auricle distance (mm)	DBH ^{a)}	Developmental criteria
Pollen mother cell	700–900	–40 to –30	14	Relatively large in size, callose wall starts to form
Tetrad	800–950	–35 to –25	13–13.5	Four microspores enclosed in callose wall
Early young microspore	1000–1200	–10–0	11	Nonvacuolate microspores just released from tetrad, germination pore is not visible
Middle young microspore	1350–1500	40–55	8	Vacuolate microspore, germination pores are visible
Early binucleate	1650–1800	70–95	5	Binucleate, pollen starts to accumulate starch
Late binucleate	1800–2000	80–125	3	Pollen starch grains begin to accumulate, pollen become engorged
Heading stage	1800–2000	110–150	0	Trinucleate, pollen grains completely starch filled, stigma become very pink in colour

a) DBH, days before heading

of anthers located at top primary branch of the panicle to the occurrence of heading (data not shown). The anther samples were taken at 5 and 3 days before heading to obtain anther material which represented vacuolated binucleate and starch-filled binucleate microspore stages, respectively.

3.2. 2-DE analysis

The proteome maps in the pH range of 4–7 were established for six discrete microspore developmental stages. The proteome maps for the pollen mother cell stage

(PMC), five days before heading (5DBH) and heading (H) are shown in Figs. 2, 3 and 4. Good reproducibility and resolution were achieved on silver stained gels. Approximately 2500 spots were detected by image analysis across the pH range 4–7. The proteome map of pollen mother cell stage was designated as our reference map and other maps were compared to the reference map to detect differentially expressed proteins. The results of gel comparison showed that more than 155 spots were found to change in amount by more than two-fold. Newly expressed low M_r proteins were detected as most abundant proteins on proteome maps of the late stage of development. The same portions of the gel regions

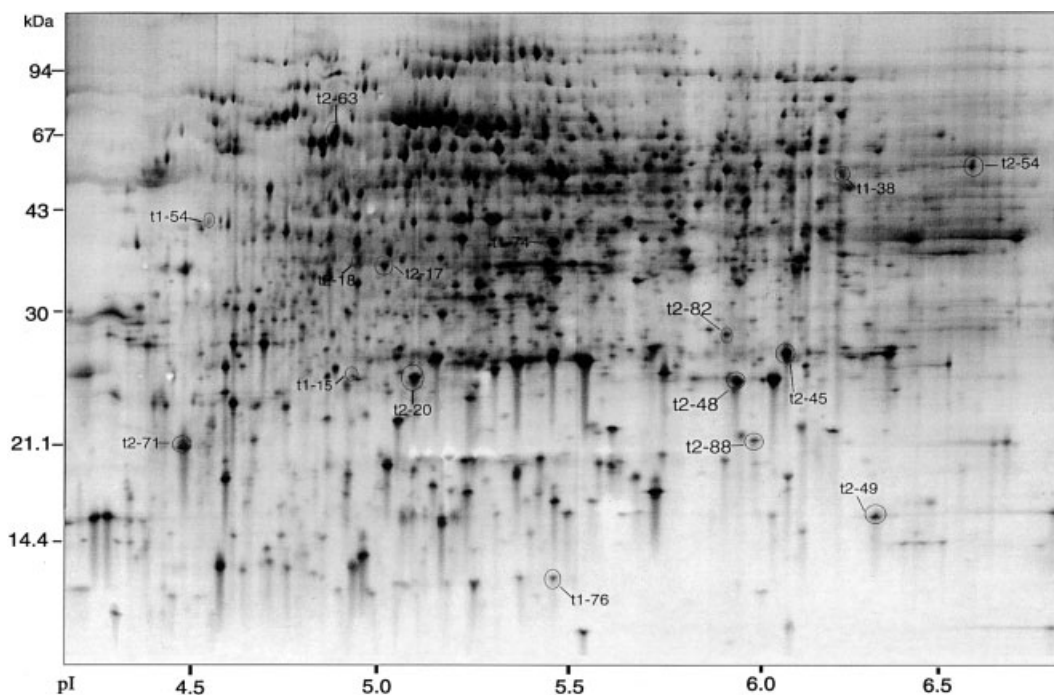


Figure 2. Representative 2-DE protein map of total anther proteins from rice cultivar Doongara at the pollen mother cell (PMC) stage. IEF in the first dimension was carried out on linear pH 4–7, 18 cm IPG strips loaded with 100 μ g of anther protein. In the second dimension a 12–14% SDS-PAGE gel was used. Proteins were visualized by silver staining. The gel regions excised for analysis are circled and the identifiers used correspond to those listed in Table 2.

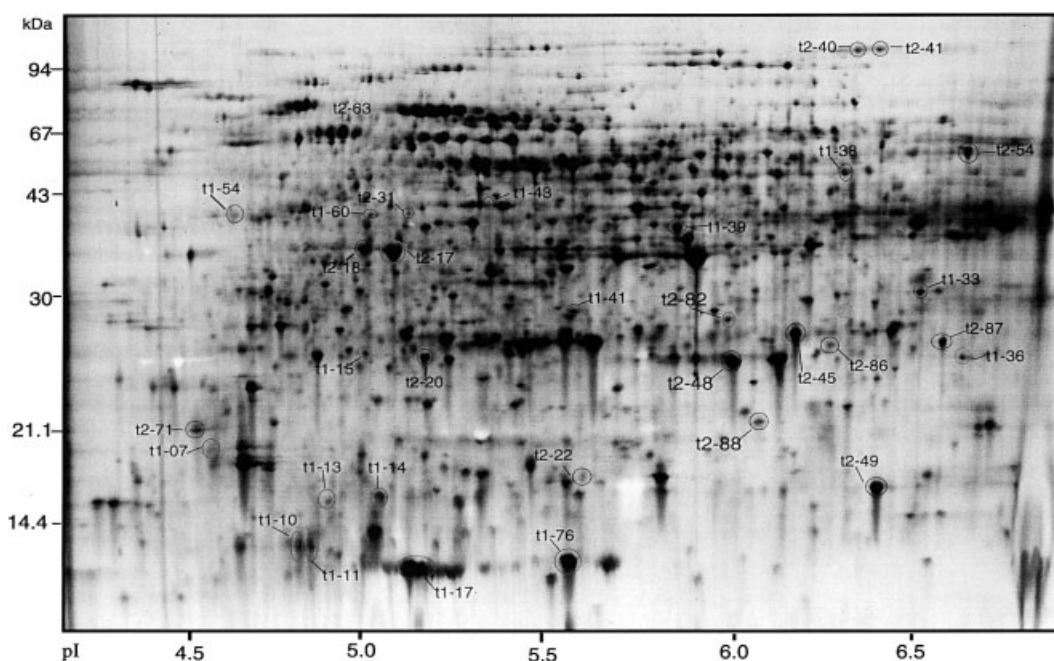


Figure 3. Representative 2-DE protein map of total anther proteins from rice cultivar Doongara at the early binucleate microspore stage. The proteins were extracted from the pooled anther materials collected 5 d before the heading stage. Gel composition, sample loading and protein visualization are as described in Fig. 2. The gel regions excised for analysis are circled and the identifiers used correspond to those listed in Table 2.

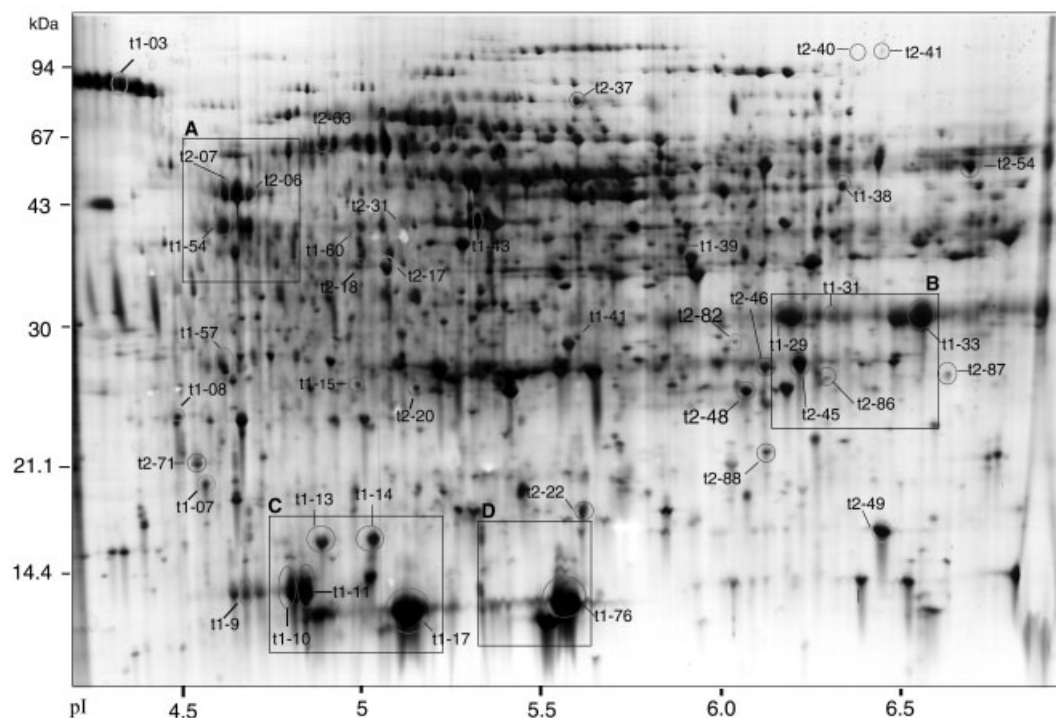


Figure 4. Representative 2-DE protein map of total anther proteins from rice cultivar Doongara at the heading (H) stage. Gel composition, loading and spot visualization is as described in Fig. 2. The analyzed spots are circled and the arbitrary identifiers correspond to those listed in Table 2. Framed areas represent the gel regions in which the protein accumulation patterns significantly change during the anther development. The enlargements of the framed areas are shown in Fig. 5.

where dramatic changes could be detected are framed in Fig. 4. The enlargements of the framed areas are shown in Fig. 5.

3.3 MS analysis and database search

In order to identify the differentially expressed proteins, 155 unique protein spots were excised from Coomassie blue G-250 stained gels and analysed by MALDI-TOF MS to generate peptide mass fingerprinting data. Some differentially displayed protein spots, which can be detected on silver gels but can not be visualized in preparative Coomassie stained gels due to the different staining properties and low abundance levels, could not be analyzed. Of the 155 mass spectrometry analyzed proteins, 10 proteins did not produce any detectable spectra due to the low amount or a composition refractive to MALDI ionization/detection [38]. The identities of 40 protein spots (26%) were assigned with high confidence by database search using PMF data. All the identified proteins were circled and assigned arbitrary numbers as shown in Figs. 2, 3 and 4. The protein identification results

by PMF analysis are shown in Table 2. Among the 40 matched database entries, 39 protein sequences are of rice origin. Approximately half of the identified protein spots are absent at the early stages of microspore development and become present or increase in amount at late stages. Four protein spots were matched against hypothetical proteins and homology searches using the amino acid sequences of these hypothetical proteins did not reveal any significant homology in the nonredundant protein databases. Another five protein spots were also matched to unknown proteins. The identified proteins are mainly involved in carbohydrate metabolism, lipid metabolism, signal transduction, cytoskeleton and cell wall formation, nucleotide and amino acid metabolism and stress responses.

3.4 N-terminal sequencing

Four late stage specific low M_r protein spots, which could not be identified with high confidence by PMF analysis, were subjected to N-terminal microsequencing analysis. Sequence data of 15 amino acid residues were obtained

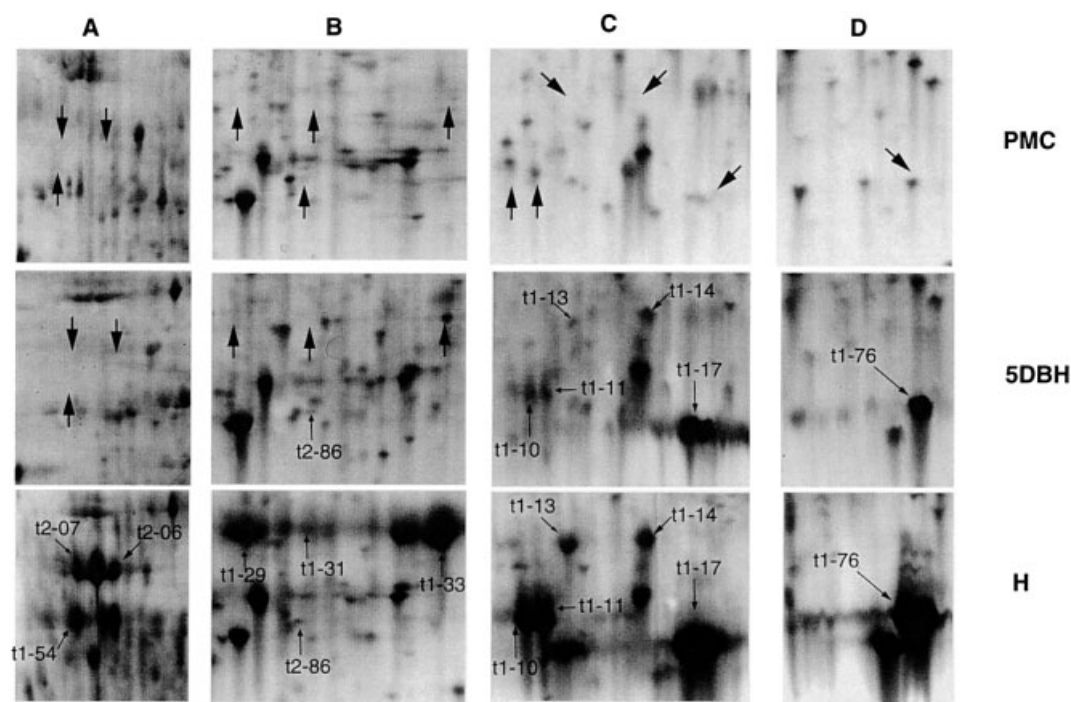


Figure 5. Enlargements of the framed regions illustrated in Fig. 4. The same areas of Figs. 2, 3 and 4 are enlarged. The arrows indicate the locations of some differentially displayed proteins, which were identified during our study. The numbers correspond to those in Table 2 and 3. PMC, pollen mother cell stage; 5DBH, 5 days before heading; H, heading.

Table 2. Results of protein identification by peptide mass fingerprinting analysis

Spot no.	Protein match to	Organism	Access. No.	Peptide number	Coverage	$M_r/pI^{(a)}$	Spot regulation ^(b)					
							PMC	EYM ^(c)	8DBH	5DBH	3DBH	H
t2-06	Vacuolar acid invertase	<i>Oryza sativa</i>	AAF87245	8	16.87%	71.3/5.1	–	–	–	+	+	+++
t2-07	Vacuolar acid invertase	<i>Oryza sativa</i>	AAF87245	10	21.00%	71.3/5.1	–	–	–	–	+	+++
t2-17	Putative fructokinase II	<i>Oryza sativa</i>	AAL26573	14	49.00%	35.9/5.0	+	+	+	++	+++	+
t2-18	Putative fructokinase II	<i>Oryza sativa</i>	AAL26573	17	49.00%	35.9/5.0	+	+	+	++	+++	+
t2-20	Submergence induced protein 2A	<i>Oryza sativa</i>	AAC19375	6	37.00%	23.65/5.1	++	++	++	++	++	+
t2-22	Hypothetical protein	<i>Oryza sativa</i>	BAA87854.1	5	40.68%	N/A	+	+	+	+	+	++
t2-31	Jab1 protein	<i>Oryza sativa</i>	AAC33765	9	38.61%	40.0/5.2	+	+	+	+	+	++
t2-37	Putative reductase	<i>Oryza sativa</i>	AAL58200	15	34.00%	82.18/5.9	–	–	–	–	–	+
t2-40	Putative beta-galactosidase	<i>Oryza sativa</i>	AAL31090	14	24.00%	92.72/6.0	–	–	–	+	++	–
t2-41	Putative beta-galactosidase	<i>Oryza sativa</i>	AAL31090	14	24.00%	92.72/6.0	–	–	–	+	++	+
t2-45	Glutathione S-transferase II	<i>Oryza sativa</i>	AAC64007	5	28.84%	23.9/6	+	+	+	+	++	++

Table 2. Continued

Spot no.	Protein match to	Organism	Access. No.	Peptide number	Coverage	$M_r/pI^{a)}$	Spot regulation ^{b)}					
							PMC	EYM ^{c)}	8DBH	5DBH	3DBH	H
t2–46	Putative soluble inorganic pyrophosphatase	<i>Oryza sativa</i>	AAK98675	5	32.00%	22.2/5.7	–	–	–	–	+	+
t2–48	beta 1 subunit of 20S proteasome	<i>Oryza sativa</i>	BAA96834	9	43.90%	26.2/5.7	++	++	+	++	++	+
t2–49	Nucleoside di-phosphate kinase	<i>Oryza sativa</i>	Q07661	4	32.89%	16.8/6.30	+	+	+	++	++	++
t2–54	Putative mitochondrial processing peptidase alpha-II chain precursor	<i>Oryza sativa</i>	BAA99436	10	23.39%	90.2/8.7	+	+	+	+	++	++
t2–63	Protein disulfide isomerase	<i>Oryza sativa</i>	BAA92322	13	52.35%	33.4/4.8	+	++	++	++	+	+
t2–71	Translationally controlled tumour protein homologue	<i>Oryza sativa</i>	P35681	8	58.33%	18.9/4.5	+	+	+	++	++	+
t2–82	Beta-ketoacyl-ACP synthase	<i>Hordeum vulgare</i>	CAA84022	5	22.63%	53.1	+	+	++	+	+	+
t2–86	GSH-dependent dehydroascorbate reductase1	<i>Oryza sativa</i>	BAA90672	7	53.99%	23.5/5.9	+	+	+	+	++	+
t2–87	Putative glutathione S-transferase	<i>Oryza sativa</i>	AAG32476	4	30.77%	24.1/6.6	++	++	++	++	+	+
t2–88	Unknown protein	<i>Oryza sativa</i>	BAA90363	5	36.87%	20.9/8.3	+	+	+	+	++	+
t1–3	Hypothetical protein	<i>Oryza sativa</i>	TC50154	4	10.78%	85.9	–	–	–	–	+	+
t1–07	Hypothetical protein	<i>Oryza sativa</i>	BAB62626	5	23.64%	42.9	–	–	+	+	+	+
t1–8	Unknown protein	<i>Oryza sativa</i>	BAA92963	8	14.05%	119.8	–	–	–	–	–	+
t1–9	Profilin	<i>Oryza sativa</i>	Q9FUD1	3	44.00%	14.24/4.9	–	–	–	–	+	++
t1–10	Profilin	<i>Oryza sativa</i>	Q9FUD1	3	44.00%	14.24/4.9	–	–	+	+	++	+++
t1–11	Profilin	<i>Oryza sativa</i>	Q9FUD1	3	44.00%	14.24/4.9	–	–	–	+	++	+++
t1–15	Lycopersicon esculentum resistance complex protein l2C-2 like protein	<i>Oryza sativa</i>	BAB07969.1	11	12.15%	164.6	+	+	+	++	++	++
t1–29	Beta expansin	<i>Oryza sativa</i>	AAF72983.1	5	29.00%	29.12/6.4	–	–	–	–	+	+++
t1–31	Beta expansin	<i>Oryza sativa</i>	AAF72983.1	4	15.00%	29.12/6.4	–	–	–	–	+	+
t1–33	Beta expansin	<i>Oryza sativa</i>	AAF72983.1	8	32.00%	29.12/6.4	–	–	–	+	+	+++
t1–36	Putative choline-phosphate cytidylyl-transferase	<i>Oryza sativa</i>	TC49579	4	25.76%	37.5	–	–	+	+	+	–
t1–38	Aminotransferase-like protein	<i>Oryza sativa</i>	AAK11219.1	4	15.53%	53.1	+	+	+	+	++	++
t1–39	Putative legumin-like protein	<i>Oryza sativa</i>	BAB63836.1	8	42.18%	40.2/5.6	–	–	–	+	++	+
t1–41	Unknown protein	<i>Oryza sativa</i>	BAB40177	4	16.27%	44.9	–	–	–	–	–	–
t1–43	Actin	<i>Oryza sativa</i>	P13362	6	24.00%	42.18/5.3	–	+	+	++	+++	+++
t1–54	Hypothetical protein	<i>Oryza sativa</i>	BAB40057.1	8	22.44%	56.2	+	+	+	+	++	++++
t1–57	Unknown protein	<i>Oryza sativa</i>	BAA96146	6	46.33%	23.59/4.7	–	–	–	–	–	+
t1–60	Unknown protein	<i>Oryza sativa</i>	BAB39912.1	5	12.48%	67.6	–	–	–	–	–	+
t1–74	Lipase-like protein	<i>Oryza sativa</i>	CAC39051.1	6	21.32%	58.8	+	+	–	–	–	–

a) molecular weight and pI of matched protein

b) – indicates that the protein spot is absent, + indicates that the spot is present, ++ indicates more than two-fold increase, +++ indicates more than five fold increase and ++++ indicates more than ten-fold increase.

c) early young microspore

for each of the four proteins analyzed. The *N*-terminal sequence and database search results are shown in Table 3. A polymorphism was observed at residue 14 for protein spot t1–17, both serine and threonine being detected in a 0.5 to 0.5 ratio. The separation of the same protein sample in 24 cm strips showed the existence of two distinct protein spots in the position of spot t1–17 (data not shown). For protein spot t1–14 two sets of sequences were obtained. One set of sequences is 100% identical to the *N*-terminal sequence of spot t1–13, which possesses the same *M_r* but has a different *pI*. This indicates that spot t1–14 is possibly a mixture of a charge isoform of spot t1–13 and another protein. FASTA homology searching of the nonredundant protein databases using the *N*-terminal sequences did not result in any significant matches. Then BLAST searches were conducted using the theoretical six-reading frame translation products of rice tentative consensus (TC) sequences stored at the TIGR Rice Gene Index database. Spots t1–76, t1–17 and t1–13 showed 100% identity to 15 amino acids in the appropriate reading frame translation products of TC sequences TC63294, TC56947 and TC57115, respectively. One set of the *N*-terminal sequences of spot t1–14, KEPETLEEEVRAGCK, showed 93% (14 in 15 amino acid residues) identity to the translation product of TC57311. BLAST homology searching of the nonredundant protein database using the theoretical translation product of matching ORFs showed that spots t1–76 and t1–17 are homologous to group II allergen proteins. As this homology includes important conserved pollen allergen domains in group II proteins, we have designated t1–17 and t1–76 as putative group-II pollen allergens of rice. Spots t1–13 and t1–14 did not give any significant match in the database. Putative signal peptide cleavage sites were detected just before the matching *N*-terminal sequences for all four theoretical translation products.

The calculated *M_r*s and *pI*s of the signal peptide excluded translation products of the matching ORFs were in close agreement with those experimentally observed.

4 Discussion

In order to resolve and analyze only the male gametophyte specific proteins, we collected and used anther materials instead of using the whole flower. The whole flower of rice, or the panicle, contains pollen grains belonging to different developmental stages. In addition, the panicle also contains many different types of somatic tissues and the female gametophyte organs. By excluding all the nonanther specific parts of panicle, the scope of our study was focused only on the anther-specific proteins, and at the same time some anther-specific and low abundant proteins were enriched by this sample prefractionation step. The anther samples collected for six discrete developmental stages covered the majority of the male gametophyte developmental process, ranges from the pollen mother cell to the mature pollen. Comparison of the 2-DE proteome maps revealed over 155 proteins whose expression levels are developmentally regulated. By integrating information about the regulation and biological function of the identified proteins, we were able to detect some of the metabolic and signalling pathways that are active in the developing rice anthers.

4.1 Jab1 and 20S proteasome

In our study we identified the two main regulators of light mediated signal transduction in plants. The protein spot t2–31 was identified as Jab1, or c-Jun activating binding protein, of animals. Jab1 is also known as COP9 (consti-

Table 3. Proteins analyzed by *N*-terminal microsequencing and FASTA search

Spot I.D.	<i>M_r</i> / <i>pI</i> ^{a)}	<i>N</i> -terminal sequence	Homo-logy (%) identity)	Organism	<i>M_r</i> / <i>pI</i> ^{b)}	Putative identity	TIGR accession number
t1–76	11000/5.6	TEVTFKVGEGSSGKS	100%	<i>Oryza sativa</i>	10085.35/5.45	Putative group II allergen	TC63294
t1–17	11000/5.2	TELTFKVAEGSSASS	100%	<i>Oryza sativa</i>	10140.43/5.12	Putative group II allergen	TC56947
t1–14	16500/5.0	KEPETLEEAVRAGCK	93%	<i>Oryza sativa</i>	12205.79/5.03	Not assigned	TC57311
t1–13	16500/4.9	TEEKDIEEAVCSEHC	100%	<i>Oryza sativa</i>	11371.72/4.85	Not assigned	TC57115

a) The observed molecular mass and isoelectric point 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

tutive photomorphogenesis 9) signalosome subunit 5 (CSN5) in *Arabidopsis*, which is a core component of the COP9 signalosome regulatory complex [39, 40]. The COP9 signalosome is composed of eight subunits that are highly conserved between plants and animals. Until recently the original names for the COP9 signalosome subunits were largely unrelated. A unified subunit nomenclature was adopted recently and the eight subunits are named CSN1 to CSN8 [41]. The COP9 signalosome is a pleiotropic regulator that is involved in numerous pathways. In plants, COP9 signalosome is a master repressor of photomorphogenesis. It can repress the expression of light-induced genes in the absence of light by interacting with 26S proteasome [42]. The presence of COP9 signalosome is necessary for the 26S proteasome to recognise and degrade HY5, a positive regulator of light induced genes, in an ubiquitin-dependent manner. The association of the 19S regulatory protein complex with the 20S proteasome, of which the beta 1 subunit was identified in this study, creates the 26S proteasome. The other subunits have been identified in rice anthers in our previous work [23]. Comparison of the proteome maps showed that the expression of the Jab1/CSN5 homologue in rice anthers is up regulated at the panicle heading stage, indicating the possible role of COP9 signalosome-mediated light signal sensing in anther dehiscence at the flowering stage.

4.2 Carbohydrate metabolism

We identified three key enzymes of the starch and sucrose metabolism pathway: vacuolar acid invertase (EC:3.2.1.26), fructokinase (EC:2.7.1.4) and β -galactosidase (EC:3.2.1.23). The gel comparison results show that the two charge isoforms of vacuolar acid invertase (spots t2–6 and t2–7) are expressed at a very low level and not visualized in Coomassie stained gels (data not shown) until 5 days before heading. Then they start to accumulate and reach a very high level just before the heading stage (Fig. 5). A similar pattern of up-regulation of spots t2–17 and t2–18, which match to fructokinase with very high confidence, was also observed starting from 5DBH continuing to the 3DBH stage. The up-regulation of these two key enzymes coincides with increased starch accumulation activity in the developing pollen grains. The developing anther is a photosynthetically inactive organ (sink). Sucrose, which is channelled from the photosynthetically active organs (source) to the anther tissues, is the main source of both the carbon and energy for the starch synthesis in pollen grains [43, 44]. The vacuolar acid invertase hydrolyses the transported sucrose into the two monosaccharides (sucrose + H₂O $\rightarrow$ glucose + fructose). The enzyme fructokinase catalyses the irreversible reaction of fructose phosphorylation (fructose + ATP $\rightarrow$ fructose

6-phosphate + ADP). The phosphorylated fructose and the glucose are further converted into glucose 6-phosphate, from which the starch is synthesized in amyloplasts, or energy is produced by the glycolysis pathway. These two reactions are very important in sink tissues where sucrose assimilation and its conversion to starch are in progress. Beta-galactosidase (spots t2–40 and t2–41) is also found to be up-regulated during the late stages. Beta-galactosidase produces galactose by hydrolysing the terminal galactosyl residues from carbohydrates, glycoproteins and galactolipids. The free galactose may enter the glycolysis pathway and release energy [45].

In addition to the major sucrose-partitioning role of the vacuolar acid invertase, the enzyme also has a proposed function in osmoregulation and cell enlargement in plants [46]. The increased osmoregulation activity of the vacuolar acid invertase may play a crucial role in anther dehiscence, mediated by changing water potential in anther tissues. The cell enlargement function is important for pollen grain engorgement and subsequent pollen tube growth after pollen release. Plant beta-galactosidase also has important roles in seed germination, fruit softening and senescence by hydrolysing the galactose-containing cell wall polymers, such as arabinogalactan, that contribute to the rigidity of the plant cell wall [45, 47]. It is possible that increased beta-galactosidase activity during the late stage of pollen development plays important roles in pollen wall loosening essential for pollen engorgement and in anther dehiscence. In our study at least two charge isoforms for each of the three enzymes mentioned above were detected on the 2-DE gels. Slight differences were observed between the peptide mass spectra profiles of each pair of isoforms. The types and the positions of the post-translational modifications giving rise to the isoforms, and the physiological effects of formation of these isoforms in the complex carbohydrate metabolism pathways, need to be further investigated.

4.3 Cell wall and cytoskeleton associated proteins

The temporal regulation patterns of some cell cytoskeleton and cell wall associated proteins clearly show that cell expansion activity increases at late microspore developmental stages. We identified three isoforms of profilin, three isoforms of β -expansin and one isoform of actin. Actin is the major component of microfilaments in eukaryotic cells. The remodelling of the cell cytoskeleton by polymerisation and depolymerisation of actin filaments is the major driving force of cytoplasmic streaming, cytokinesis, cell expansion and development [48, 49]. Profilin is a small actin monomer-binding protein (usually 12–15 kDa)

which can have two opposing effects on actin polymerisation. By sequestering G-actin, profilin can cause the depolymerisation of actin microfilaments, or it can promote actin polymerisation by stimulating the exchange of ADP for ATP on G-actin [50]. Recent studies showed that profilins are involved in the signalling pathways that regulate pollen tube growth by modulating protein kinase and phosphatase activity in pollen [51, 52]. Ramachandran *et al.* [48] also reported that the expression levels of profilin could affect flowering time in *Arabidopsis*. Many research findings indicate the importance of profilin in pollen development. In our study, the accumulation of profilin isoforms (spots t1–9, t1–10 and t1–11) gradually increased starting from the first pollen mitosis and reached the highest level at the heading stage. The temporal regulation patterns of profilin and actin are paralleled by cell activities, such as cell division and cell elongation, in which the reorganization of the actin cytoskeleton is crucial. This suggests the active involvement of these proteins in cell cytoskeleton remodelling during the two rounds of pollen mitosis, cell enlargement and pollen tube growth during the pollen germination. The chemical nature of the different profilin isoforms and their potential role in signal transduction pathways during male gametophyte development needs to be further studied.

We also identified three isoforms of β -expansin (spots t1–29, t1–31 and t1–33), which are expressed only at late stages. It has been demonstrated that β -expansins, also known as group I grass allergens, can induce the cell wall loosening necessary for plant cell growth, cell division and cell separation [53]. However, there is still controversy over the cell wall loosening mechanism of β -expansins [54–57]. In developing pollen, the β -expansins may play two roles. First, the expression of the β -expansins are up-regulated to meet the increasing demand of cell wall loosening during the mitosis and the pollen engorgement during the late stage of pollen development. Second, the accumulated β -expansins can aid the penetration of the growing pollen tube through the stigma and style by loosening the style cell walls after the pollen release. We observed a very similar temporal regulation pattern for both the β -expansins and profilins. It is possible that the pollen cytoskeleton re-modelling and the accompanied cell wall expansion activities during the pollen tube growth are controlled by the coordinated regulation mechanism of profilin and β -expansin. Baluska *et al.* [58] has reported the association of these two proteins in tip growth of root hair formation.

4.4 New ORFs are defined

Currently the new EST and the genomic sequence information databases are expanding very fast. Although the

advances in bioinformatics and the accumulation of vast genomic sequence information make it possible for comparative genomics to predict and annotate the ORFs from EST or genomic sequence, the success rate of correct prediction and annotation is not very high [20]. This is particularly evident in the case of small ORFs. Even if a gene is successfully predicted and annotated by bioinformatics tools, the validation of the prediction by other protein based experimental approaches is necessary. In our study, the *N*-terminal amino acid sequences obtained for four low M_r proteins matched to four unannotated EST sequences in the public database. These ORFs were not correctly predicted, possibly because of their small size. The conformation of four hypothetical proteins by PMF analysis of four other protein spots verified the correct prediction of these genes and their expression in the anther tissue. The results of our *N*-terminal microsequencing and data analysis clearly demonstrate that 2-DE based proteomics is an ideal complementary tool to the comparative genomic analysis in gene prediction.

The PMF analysis of protein spots t1–17, t1–76, t1–13 and t1–14 did not produce the identifications we obtained after *N*-terminal analysis. Low M_r protein spots produce after tryptic digestion only a few peptides in the spectra-acquiring mass range (600–3500 Da) of mass spectrometry analysis. Furthermore, not all the tryptic peptides generated can necessarily be ionized and detected. These factors reduce the number of experimental peptide masses available to produce confident identification of small proteins by PMF analysis. From the results of PMF and *N*-terminal sequencing analysis of these four protein spots, we concluded that *N*-terminal sequencing is more suitable for the identification of unknown low molecular size proteins than PMF analysis.

5 Concluding remarks

In this study we successfully employed the proteome analysis technique as a tool to investigate the global protein expression profiles of male gametophyte development in rice. Our results have provided useful information about the protein complement of rice anthers and its temporal regulation during pollen development. It is almost certain that some low-abundant proteins, which may play very important signalling and regulatory roles during the male gametophyte development, were not visualized in our 2-DE gels and therefore not included in our study. With rapid advances in protein fractionation and separation technology we will in the future be able to visualize and analyze those important proteins. It is possible that we could not identify all of the differentially

displayed proteins by PMF analysis because the genes coding for all of these proteins are probably not yet represented in the databases. The 26% success rate with protein identification by PMF analysis compares to the 68% success rate of protein identification by PMF in maize leaf proteome analysis [59] and the 55% of success rate by *N*-terminal sequence matching in wheat endosperm proteome analysis [60]. The draft version of the rice genome has been released but the annotation of the genome is still in progress. The completion and the release of the fully annotated rice genome should enable us to identify most of the anther specific proteins and eventually it will lead to a better understanding of the molecular mechanism underlying male gametophyte development in plants.

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