Proteomics Identification of Differentially Expressed Proteins Associated with Pollen Germination and Tube Growth Reveals Characteristics of Germinated *Oryza sativa* Pollen*[S]

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Mature pollen from most plant species is metabolically quiescent; however, after pollination, it germinates quickly and gives rise to a pollen tube to transport sperms into the embryo sac. Because methods for collecting a large amount of *in vitro* germinated pollen grains for transcriptomics and proteomics studies from model plants of *Arabidopsis* and rice are not available, molecular information about the germination developmental process is lacking. Here we describe a method for obtaining a large quantity of *in vitro* germinating rice pollen for proteomics study. Two-dimensional electrophoresis of ~2300 protein spots revealed 186 that were differentially expressed in mature and germinated pollen. Most showed a changed level of expression, and only 66 appeared to be specific to developmental stages. Furthermore 160 differentially expressed protein spots were identified on mass spectrometry to match 120 diverse protein species. These proteins involve different cellular and metabolic processes with obvious functional skew toward wall metabolism, protein synthesis and degradation, cytoskeleton dynamics, and carbohydrate/energy metabolism. Wall metabolism-related proteins are prominently featured in the differentially expressed proteins and the pollen proteome as compared with rice sporophytic proteomes. Our study also revealed multiple isoforms and differential expression patterns between isoforms of a protein. These results provide novel insights into pollen function specialization. ***Molecular & Cellular Proteomics 6:207–230, 2007.***

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Pollen of flowering plants, generated in diploid sporophytic plants via meiosis followed by two cycles of mitosis, contains three haploid genomes and is a highly reduced organism. Mature pollen grains from most plant species are metabolically quiescent. However, during pollination, they can quickly germinate and give rise to a polarly growing pollen tube whereby the pollen interacts with pistils and then delivers two sperms into the embryo sac to initiate double fertilization. Besides having biological importance, pollen germination and tube growth have been considered unique developmental processes for studying cell polar establishment, cell differentiation, cell fate determination, and cell-to-cell recognition. Thus, the molecular mechanisms underlining the specific cellular programs have been the focus of investigation over the past 50 years (1). However, until now, only a limited number of genes encoding coat/wall proteins or signal molecules have been shown to be essential for pollen germination, tube growth, and interaction of the tube and stigma (1–7).

Recent analyses of mature pollen of *Arabidopsis* revealed the transcriptome to have reduced complexity and a higher proportion of selectively expressed transcripts than sporophytic tissues (8–10). As well, about one-third of the genes expressed in vegetative tissues are not expressed in the pollen (8). The observation suggests that the transcriptional characteristics involve pollen function specialization. Furthermore these studies determined that pollen transcriptome has a functional skew toward transcripts implicated in cell wall metabolism, signaling, and cytoskeletal dynamics. These results also support the early notion that mature pollen has stored presynthesized mRNAs (11) and present a novel insight into pollen function specialization at the whole genome level.

However, the transcriptomic data of *Arabidopsis* pollen showed that transcripts related to translation and glycolysis/energy metabolism were underrepresented in pollen (8–9). All transcripts encoding putative or known ribosomal proteins seem to be undetectable in the transcriptome (9). Given the ability of pollen to initiate protein synthesis rapidly upon germination and the carbon skeleton/energy needs for active

tube growth, the transcriptome data raise questions, although these results might be explained by the proteins related to the functional categories being presynthesized during the early stage of male gametophytic development (8).

Recent proteomics studies from *Arabidopsis* (12, 13) and rice (14) have revealed that mature pollen presynthesizes a complement of proteins required for pollen function, including those implicated in protein synthesis and carbohydrate/energy metabolism. Dai *et al.* (14) showed that these functional categories related to carbohydrate/energy metabolism, wall metabolism, protein synthesis and degradation, and signaling are overrepresented in the proteome. This finding indicated that proteomic data are a necessary complement to transcriptomic data of pollen and are essential to our understanding of pollen function. Importantly proteomics analyses showed that mature pollen contains multiple isoforms; this suggests that posttranslational modifications seem to be crucial to pollen function.

In contrast to our increased understanding of molecular information in mature pollen, our knowledge of genome-scale events underlying germination and fast tube growth are still lacking, although these data are essential for understanding the molecular mechanisms involved in fast tube growth and invasion in pistils. Such knowledge will help dissect the molecular regulation of sexual reproduction and important cellular programs such as polarized cell growth and cell recognition. Recently Fernando (15) compared 2-D[1] electrophoresis (2-DE) protein maps of mature and germinated pollen from the gymnosperm *Pinus strobus*, which produces unlimited quantities of pollen grains, the pollen having *in vitro* germination activity over an extended period. The author detected 57 protein spots that were specifically expressed or increased in expression in the germinated pollen. However, because of a lack of genomic sequence information about the species, 33% of these spots were not identified.

Arabidopsis and rice have been accepted as the model plants of dicots and monocots of angiosperms, respectively. They have small genome size, and the two genomes have been completely sequenced. Transcriptomic and proteomic data about pollen tubes will greatly increase our knowledge about tube development. Several efforts have established an *in vitro* germination system for *Arabidopsis* pollen (16), but collecting enough germinated pollen grains from the plant is difficult. In contrast, we can collect a relatively large amount of mature pollen grains from rice, but a main difficulty in using rice is that the mature pollen quickly loses the ability to germinate under *in vitro* conditions after being released from anthers (17). Therefore, some technological breakthroughs are required for proteomics and transcriptomics studies of germinated pollen from the two model plants.

Inhibitor experiments have shown that early tube growth strictly depends on protein synthesis that is relatively independent of transcription (11). So proteomics identification of proteins differentially expressed in mature and germinated pollen will generate important molecular information. In the present study, we wanted to address the difference between proteome maps of mature and germinated pollen, which proteins are synthesized during tube growth, and the functional characteristics of newly synthesized proteins. We first established an *in vitro* germination system of rice pollen, and then used 2-DE followed by MALDI-TOF MS and ESI-Q-TOF MS/MS to identify 160 differentially expressed proteins (representing 120 unique proteins) associated with germination and tube growth. These proteins were implicated in 12 groups of known function and one of unknown function with obvious functional skew toward carbohydrate/energy metabolism, wall metabolism, protein synthesis and degradation, cytoskeleton dynamics, and stress response. Of the differentially expressed unique proteins, 25% had isoforms, and isoforms of some protein species showed distinct changes in expression. Our results suggest that phosphorylation and glycosylation are involved in the generation of these isoforms.

MATERIALS AND METHODS

Collection and in Vitro Germination of Mature Rice Pollen Grains (MPGs)—Rice cultivar Zhonghua 10 (*Oryza sativa* L. ssp. *japonica*) was planted under a natural growth season in Beijing (39° 54′ N, 116° 24′ E). The plants were managed as usual. MPGs were collected by shaking panicles gently during anthesis. These collected fresh MPGs were transferred into a liquid germination medium (20% sucrose, 10% polyethylene glycol 4000, 3 mM $Ca(NO_3)_2 \cdot 4H_2O$, 40 mg/liter H_3BO_3, 3 mg/liter vitamin B1) and cultured for about 10 min at room temperature (~30 °C) to generate synchronously germinated rice pollen grains (GPGs). The amount of GPGs was examined under a microscope (also see below). After anther debris were removed by filtering the pollen culture through cheesecloth, GPGs were collected by centrifuging at 500 × *g*. MPGs and GPGs were used immediately for extracting proteins or stored at −80 °C until use.

Observation of Pollen Morphology—Morphological characteristics of MPGs were examined under a microscope (Axioskop 40 fluorescence microscope, Zeiss) by staining with 1% I_2-KI, 1% triphenyltetrazolium chloride in 50% sucrose or 0.2 μg/μl 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes). The GPGs stained with DAPI or FM 1–14 (0.2 μM; Molecular Probes) were observed by microscopy with a confocal laser scanning microscope (Zeiss) or Axioskop 40 fluorescence microscope.

Preparation of Proteins from MPGs and GPGs—MPGs and GPGs were homogenized in a homogenate buffer (50 mM Tris-HCl, pH 7.5, 20 mM KCl, 2% Nonidet P-40, one tablet of protease inhibitor mixture/25 ml (Roche Applied Science), 13 mM DTT) with use of a chilled mortar and pestle. Supernatant was collected by centrifugation at 18,000 × *g* for 20 min at 4 °C and then supplemented with trichloroacetic acid to a final concentration of 12.5% to precipitate proteins on ice for 2 h. Proteins were pelleted by centrifugation at 15,000 × *g* for 20 min at 4 °C and then resuspended in 80% cold acetone containing

[1] The abbreviations used are: 2-D, two-dimensional; 2-DE, 2-D electrophoresis; MPG, mature rice pollen grain; GPG, germinated rice pollen grain; DAPI, 4′,6-diamidino-2-phenylindole; MM, molecular mass; CBB, Coomassie Brilliant Blue; RV, relative volume; PMF, peptide mass fingerprinting; Unipros, unique proteins; POD, peroxidase; TCTP, translationally controlled tumor protein; MT, microtubulin; PTM, posttranslational modification; VDAC, voltage-dependent anion channel protein; eIF, eukaryotic initiation factor.

0.07% β-mercaptoethanol. The mixture was placed at −20 °C for 30 min to allow proteins to precipitate, and proteins were collected by centrifugation at 15,000 × *g* for 20 min at 4 °C. After being rinsed with cold acetone with 0.07% β-mercaptoethanol and dried by vacuum, the resulting proteins were dissolved in a lysis buffer (7 M urea, 2 M thiourea, 4% Nonidet P-40, 13 mM DTT, 2% Pharmalyte 3–10) and used for 2-DE immediately or stored in aliquots at −80 °C after debris were removed by centrifugation at 20,000 × *g* for 20 min at 4 °C. For protein preparation of MPGs or GPGs, triplicate biological samples were used. Protein concentrations were determined according to the Bradford method (18) by DU640 UV-visible spectrophotometry (Beckman). Bovine serum albumin was used as the standard.

2-DE Gel Staining and Image Analysis—An aliquot (about 600 μg of proteins) of protein sample prepared from MPGs or GPGs was diluted with rehydration buffer (7 M urea, 2 M thiourea, 1% Nonidet P-40, 13 mM DTT, 0.5% IPG buffer 3–10, 0.002% bromphenol blue) and loaded onto an IPG strip holder of a 24-cm, pH 3–10 or pH 4–7 linear gradient IPG strip (Amersham Biosciences). Isoelectric focusing involved the Ettan IPGphor isoelectric focusing system following the protocol of the manufacturer (Amersham Biosciences). For SDS-PAGE, the equilibrated IPG gel strips were placed onto 12.5% ExcelGel SDS gels (Amersham Biosciences) by use of an Ettan DALT Six electrophoresis unit. Low molecular mass (relative molecular mass (MM)) protein markers (Fermentas) were co-electrophoresed as MM standards. The proteins in gels were visualized by Coomassie Brilliant Blue (CBB) staining. 2-DE experiments were repeated three times for each protein sample, and images were obtained by scanning each stained gel at 300 dots/inch with use of an ImageScanner (Amersham Biosciences). The apparent MM of each protein in gels was determined with the co-electrophoresed MM protein markers used as standards. An apparent pI of each protein was determined by its migration on IPG linear strips.

Protein Quantification and Expression Abundance Analysis—All 2-DE gel images were analyzed by use of the ImageMaster 2D platinum 5.0 program (Amersham Biosciences). After spot detection, quantification, and background subtraction, the relative volume (RV) of each protein spot was obtained by dividing the volume of the spot by the total volume of all spots in a gel. Furthermore to compare protein abundance between two independent datasets of each sample directly (pH 4–7 and pH 3–10), we generated a normalized RV whereby the RV of each spot was multiplied by a correction constant (*C*). Correction constants for pH 4–7 (C_{pH4-7}) and pH 3–10 (C_{pH3-10}) were calculated according to the formulas described by Hajduch *et al.* (19).

The normalized RV values of protein spots in triplicate biological repeats for each sample underwent further statistical analysis to evaluate their relative standard deviation (S.D.). These reproducible protein spots were used further to identify proteins differentially expressed in MPGs and GPGs. The significance of differentially expressed proteins between MPGs and GPGs was examined by *t* test. Proteins considered differentially expressed were those that displayed at least a 2-fold significant change in RV values (p value <0.05) between the two samples.

MALDI-TOF MS—The differentially expressed protein spots were excised from 2-DE gels by use of the Ettan Spot Cutter (Amersham Biosciences), destained in a destaining buffer (25 mM ammonium bicarbonate, 50% (v/v) acetonitrile), and then dehydrated by use of acetonitrile and spun dry. Enzyme digestions in gels were carried out in 25 mM ammonium bicarbonate buffer containing 10 ng/μl sequencing grade modified trypsin (Roche Applied Science) at 37 °C for 16 h. For MALDI-TOF MS, one aliquot of the enzyme digest solution was spotted onto a sample plate with matrix (α-cyano-4-hydroxcinnamic acid, 8 mg/ml in 50% (v/v) TFA) and allowed to air dry. MALDI-TOF MS acquisition involved use of an Autoflex MALDI mass spectrometer (Bruker Daltonics) equipped with a flight tube (reflex mode, 2.6 m long), laser (N_2, 337 nm), and scout 384 target system. Accelerating voltage was 20 kV, and the microchannel plate detector was at 1.6 kV. Mass spectra were acquired in a positive mode. To ensure the accuracy of protein identification, MALDI-TOF MS was internally calibrated with the use of the peptide calibration standard (Bruker) to reach a typical mass measurement accuracy of 100 ppm. The known trypsin-autocleavable peptide masses of 9.07 and 22.73 kDa were used for a two-point internal calibration for each spectrum.

Peptide mass fingerprintings (PMFs) were searched in the National Center for Biotechnology Information non-redundant (NCBInr) protein databases with use of the search engine MASCOT (Matrix Science). *O. sativa* was chosen for the taxonomic category. All peptide masses were assumed to be monoisotopic and $[M + H]^+$ (protonated molecular ions). Searches involved use of a mass accuracy of ±100 ppm, and one missed cleavage site was allowed for each search. The identified proteins had to be in the top hit with more than four peptides matched and a sequence coverage of more than 10%.

ESI Q-TOF MS/MS—For nanospray ESI Q-TOF MS/MS (Micromass) analysis, 1–2 μl of each digested peptide sample underwent trapping column filtration for desalting treatment before being loaded in the nanoflow probe tip (Micromass). The instrument accuracy was calibrated by the external calibration of Glu-fibrinogen (3 ppm). The applied spray voltage was 800–1000 V with a sample cone of 25–40 V. The microchannel plate detector was at 2250 V, and the energy-adjustable collision cell was filled with pure argon gas. MS/MS data processing involved use of MassLynx 3.5, and data were searched in the NCBInr protein sequence databases by use of the MS/MS ion search program MASCOT (Matrix Science). The identified proteins had to be in the top hit with more than two peptide sequences matched. Under less-than-optimal circumstances, a matched protein was accepted if it ranked as the top hit with a single peptide match. In this case (only four identities), the MS/MS fragment ion pattern was verified by manual inspection. The manual inspection of MS/MS data processing involved use of the Peptide Sequencing Program with the following strict criteria: 1) MM tolerance was set at 0.3 Da, and mass type was assumed to be monoisotopic; 2) the peak threshold was 0.15%, and the fragment ion tolerance 0.15 Da; and 3) most importantly, nearly complete Y-ion series and partial complementary B-ion series needed to be present, and the Y-ions should correspond to peaks with high relatively intensity.

Western Blot Analysis—After separation by 12.5% SDS-PAGE, proteins were electrophoretically transferred on a semidry blot apparatus to a PVDF membrane (Pierce) and then immunodetected according to the methods of Dai *et al.* (14). The primary antibodies used were against maize pollen coat-specific β-1,4-xylanase (20), pumpkin catalase 1 (21), plasma membrane H^+-ATPase PMA2 from *Nicotiana plumbaginifolia* (22), and rice OsRad21-3 (prepared in our laboratory).

Semiquantitative RT-PCR—Semiquantitative RT-PCR was used to analyze the accumulation patterns of the following transcripts in anthers, MPGs, and GPGs. Primers Pr1 (5′-ACG CGC TGA CCA AGG AGA TC-3′) and Pf1 (5′-CAG GTT GTA GTC GCC GTG TG-3′) were used to detect the transcript for β-1,4-xylanase (GenBank accession number AAP53220), primers Pr2 (5′-ACT TGG CCT GGA CGT TGG AC-3′) and Pf2 (5′-ATG GCA TCC TCC TCC CTT CT-3′) were used for β-expansin (NP_91258), primers Pr3 (5′-GCA AGC ATG CAG CAA CAC AT-3′) and Pf3 (5′-GCA GCA ATG GCA TCC TCC T-3′) were for major pollen allergen Ory s 1 (Q40638), primers Pr4 (5′-TTT GCA TAT GGC TCC ACA GC-3′) and Pf4 (5′-ATG GCC TCC ATG TCC TCC TTC-3′) were for pollen allergen 1 (CAD40508), primers Pr5 (5′-TCA GAT CAT GTT GGA GAG G-3′) and Pf5 (5′-ATG GCG AGA TCA CTG GCG C-3′) were for pectin methylesterase inhibitor (NP_912762), and primers Pr6 (5′-ACA GAG CCT GCC ACG ACA GA-3′) and Pf6 (5′-GAC CGT TGC CTT CAA TAG CG-3′) were for β-galactosidase

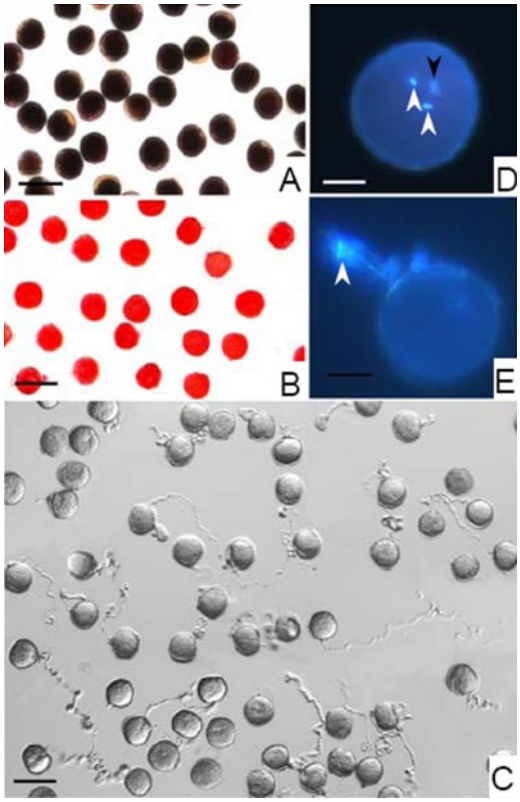


FIG. 1. **Cytological characteristics of *in vitro* GPGs.** *A* and *B*, I_2-KI-stained (*A*) and triphenyltetrazolium chloride-stained (*B*) MPGs showing metabolic activity. *C*, differential interference contrast microscopy of GPGs. *D*, DAPI-stained MPGs showing germ unit consisting of two sperm nuclei (*white arrows*) and one vegetative nucleus (*black arrow*). *E*, DAPI-stained GPGs showing transport of a germ unit (*arrow*) into the tip of a fast growing pollen tube. *Scale bar*, 50 μm in *A*, 60 μm in *B* and *C*, and 14 μm in *D*.

(NP_920740). The amplified tubulin tubA cDNA (X91806) was used as a constitutive control (23). First strand cDNA was synthesized with SuperScript II RNase H^- reverse transcriptase (200 units/μl, Invitrogen) according to the manufacturer's protocol. PCR was performed in a mixture of 50 μl that contained 1 μl of first strand cDNA, 10 pmol each of the gene-specific primers, 0.4 mM dNTPs, 1× PCR buffer, and 2.5 units of *LA Taq* DNA polymerase (5 units/μl, TaKaRa) for 25 cycles.

Detection of Glyco-/Phosphoprotein Candidates—After separation by 2-DE according to the protocol described under "2-DE Gel Staining and Image Analysis," proteins in the gel were stained with Pro-Q diamond phosphoprotein and Pro-Q Emerald 488 glycoprotein gel stain kits according to the manufacturer's protocol (Molecular Probes). Images were acquired on a Typhoon 9400 variable mode imager (Amersham Biosciences) with a 532 nm laser excitation and 560-nm bandpass emission filter for Pro-Q diamond-stained proteins and by use of 488 nm laser excitation with a 530-nm bandpass emission filter for Pro-Q Emerald 488-stained proteins. Finally the gel was stained by CBB. Unequivocal identification of glyco- and/or phosphoproteins was by grayscale adjustment of images depending on the co-electrophoresed "PeppermintStick" phosphoprotein and "CandyCane" molecular mass standards (Molecular Probes).

Bioinformatics Analysis—Protein functional domains were predicted by use of the PHI and PSI-BLAST programs (www.ncbi.nlm.nih.gov/BLAST/). Signal peptides and subcellular localization information were obtained by use of SignalP and PSORT. Chromosome loci of protein-coding genes were detected at mpss.udel.edu/rice/. Amino acid sequence identity/similarity analysis was conducted by use of DNAman software and the BLAST program (www.ncbi.nih.gov/blast/).

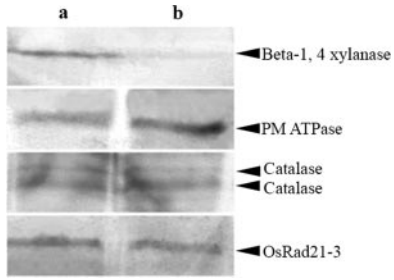


FIG. 2. **Western blot detection of four marker proteins in MPGs (*a*) and GPGs (*b*).** Proteins were separated by one-dimensional SDS-PAGE and then transferred to PVDF membranes. The membranes were immunodetected with rabbit polyclonal antibody against maize pollen coat β-1,4-xylanase (20) (AAF70549) (1:200 dilution), which shares 70% amino acid identity with its rice homolog AAP53220; plasma membrane H^+-ATPase PMA2 (22) (A43637) from *N. plumbaginifolia* (1:2000 dilution), which shares 90% amino acid identity with its rice homolog CAD29296; catalase 1 (21) (P48350) from pumpkin (1:2000 dilution), which shares 85 and 77% amino acid identity with its rice homologs XP_470174 and AAQ19030, respectively; or nucleus-localizing rice OsRad21-3 protein (25) (1:2000 dilution, antibody was prepared in our laboratory).

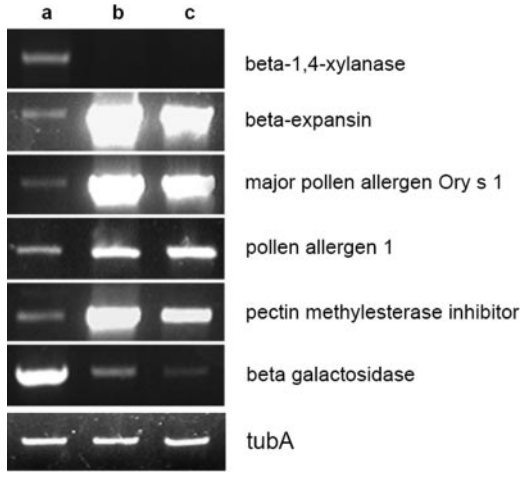


FIG. 3. **Changed level of expression of transcripts encoding six rice MPG-presynthesized proteins (14) during pollen germination and tube growth.** Semiquantitative PCR was performed with first strand cDNAs synthesized with total RNA from anthers (*a*), MPGs (*b*), and GPGs (*c*). The amplified tubA cDNA was used as a constitutive control. PCR products were separated on 1% agarose gels.

RESULTS

In Vitro Germination of Mature Rice Pollen—Rice pollen is a representative tricellular wind-pollinated pollen. It has a thinner wall (0.8–1.2 μm) and fewer lipids in the coat layer than other plants in Gramineae (14). The pollen appears to have lower viability and less longevity under *in vitro* conditions (17) than maize pollen, especially dicot pollen (24). All these char-

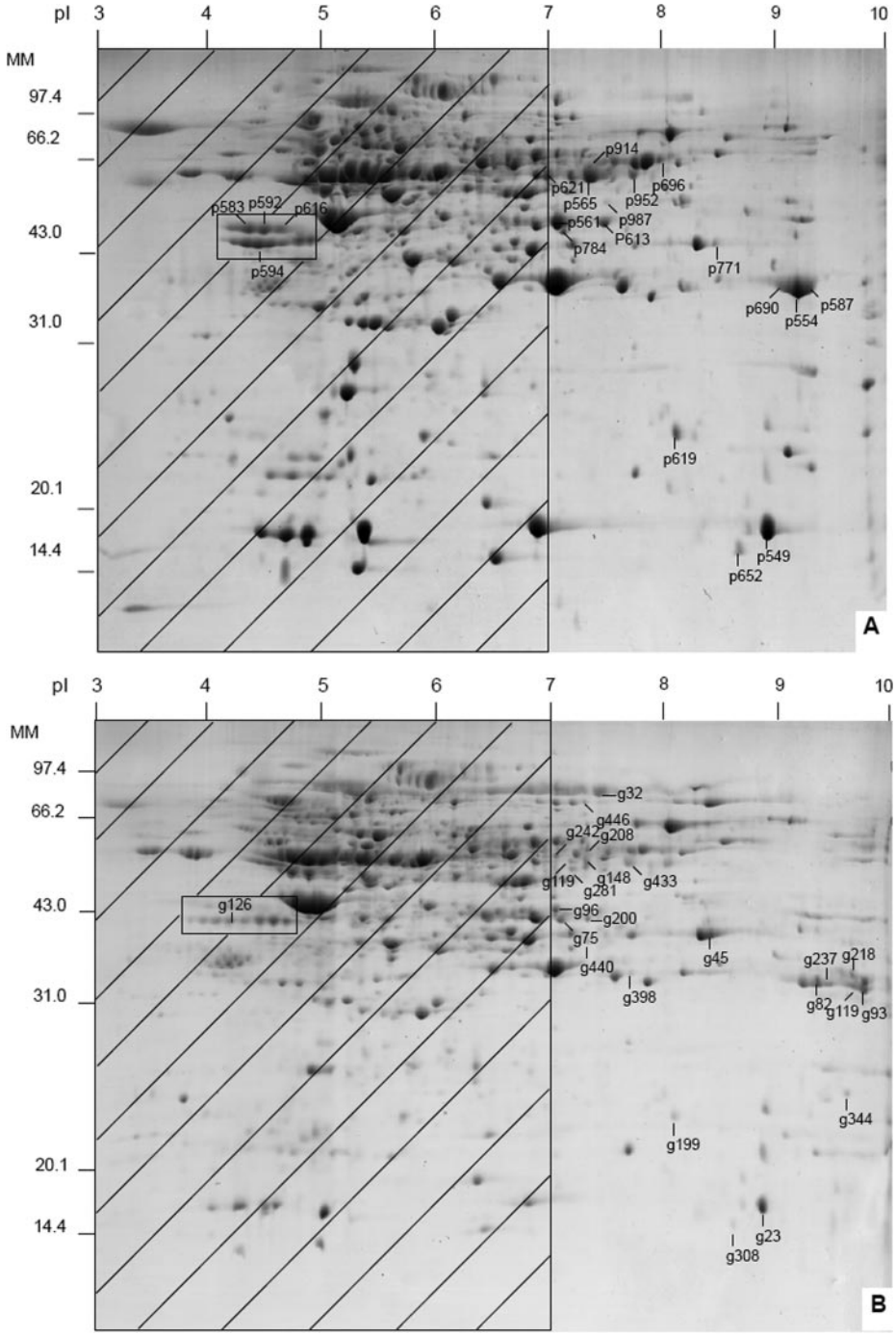


FIG. 4. **Representative 2-DE images of MPG (*A*) and GPG (*B*) proteins in the pH 3–10 range.** Proteins were separated by 2-D PAGE and stained with CBB. MM in kilodaltons and pI of proteins are indicated on the *left* and *top* of each image, respectively. *A*, a representative 2-DE image of MPG proteins. An average of 559 ± 10 spots were detected from three replicate repeats; of them, 146 ± 6 spots were found to distribute around pH 7–10. *B*, a representative 2-DE image of GPG proteins. An average of 544 ± 11 spots were detected from three replicate repeats; of them, 140 ± 5 spots were around pH 7–10. These differentially expressed protein spots in the MPG and GPG gels were matched and identified mainly from the range of pH 7–10. In the shadowed range of pH 3–7, the numbered spots were identified. Protein spots from MPGs are numbered with prefix "*p*," and those from GPGs are numbered with "*g*." Numbered spots correspond to proteins in Tables I, II, and III and Supplemental Table S1.

acteristics make it difficult to germinate rice pollen in large amounts under *in vitro* conditions (17). Our preliminary experiments showed that most MPGs of rice became inviable when stored for more than 10 min at room temperature or −80 °C (data not shown). Therefore in this study, we collected rice MPGs from blossoming flowers, immediately transferred them to a germination medium, and cultured them for a given time at room temperature (~30 °C). The collected MPGs showed an active metabolism (Fig. 1, *A* and *B*). After germination, anther debris were removed by filtering the culture, and GPGs were collected by centrifugation. Purity examination by microscopy revealed no contamination by other tissues in the filtered culture (Fig. 1*C*). This procedure gave rise to a germination ratio of more than 80% (Fig. 1*C*). The germinating pollen can transport an entire germ unit into the pollen tube (Fig. 1, *D* and *E*).

To evaluate whether the *in vitro* germinated pollen had normal levels of proteins and transcripts, we examined

TABLE I

Proteins up-regulated on pollen germination identified by 2-DE with MS

TPA, third party annotation; ARD, acireductone dioxygenase; BiP, binding protein; PLP, proteolipid protein.

IN[a]	Spot no.[b]	Protein name	Chr.[c] locus	NPV[d]	NGV[d]	NCT[e]	*p* value[f]
Wall remodeling and metabolism							
1	-/G601	UDP-glucose pyrophosphorylase	Os02g02560		0.31 ± 0.05		
2	p914/g208	Putative UDP-glucose dehydrogenase	Os03g40720	0.01 ± 0.005	0.10 ± 0.02	8.66	0.01963
3	P902/G874	Reversibly glycosylated polypeptide	Os03g40270	0.06 ± 0.01	0.19 ± 0.02	3.12	0.00231
4	P95/G35	Reversibly glycosylated polypeptide	Os03g40270	1.41 ± 0.09	3.71 ± 0.25	2.64	0.00064
5	-/G119	Reversibly glycosylated polypeptide	Os03g40270		1.44 ± 0.12		
6	P952/G268	dTDP-D-glucose 4,6-dehydratase-like, containing RfbD domain COG1091, putative dTDP-4-dehydrorhamnose reductase	Os02g45540	0.05 ± 0.01	0.64 ± 0.04	12.86	0.00206
7	-/g440	Putative *myo*-inositol-1-phosphate synthase	Os10g22450		0.01 ± 0.001		
8	P794/G339	TPA: class III peroxidase 79	Os06g27850	0.08 ± 0.01	0.52 ± 0.04	6.17	0.00247
9	P444/G245	TPA: class III peroxidase 78	Os06g20150	0.20 ± 0.03	0.71 ± 0.09	3.53	0.01106
10	P702/G699	TPA: class III peroxidase 78	Os06g20150	0.11 ± 0.02	0.26 ± 0.03	2.43	0.00544
11	P605/G326	TPA: class III peroxidase 31	Os02g50770	0.13 ± 0.02	0.54 ± 0.06	4.04	0.00761
12	P194/G45	TPA: class III peroxidase 31	Os02g50770	0.58 ± 0.06	3.29 ± 0.41	5.72	0.00762
13	-/g242	Putative exopolygalacturonase	Os02g10300		0.01 ± 0.002		
14	-/g281	Putative exopolygalacturonase	Os02g10300		0.01 ± 0.002		
15	-/g93	β-Expansin	Os10g40090		0.04 ± 0.01		
16	-/g398	Putative beta-expansin	Os03g01640		0.01 ± 0.001		
Carbohydrate and energy metabolism							
17	P240/G47	α-1,4-Glucan phosphorylase H isozyme	Os01g63270	0.45 ± 0.05	3.22 ± 0.25	7.19	0.00263
18	-/G89	α-1,4-Glucan phosphorylase H isozyme	Os01g63270		1.76 ± 0.11		
19	-/g32	α-1,4-Glucan phosphorylase H isozyme	Os01g63270		0.08 ± 0.01		
20	P175/G78	Putative sucrose-6^F-phosphate phosphohydrolase	Os01g27880	0.67 ± 0.03	2.03 ± 0.13	3.01	0.00297
21	P544/G294	Hexokinase	Os05g44760	0.16 ± 0.02	0.58 ± 0.04	3.74	0.00048
22	P39/G5	Cytoplasmic aldolase, putative fructose-1,6-bisphosphate aldolase	Os05g33380	3.33 ± 0.45	9.89 ± 1.11	2.97	0.00246
23	P73/G2	OJ000223_09.15, putative glyceraldehyde-3-phosphate dehydrogenase	Os04g40950	1.81 ± 0.20	14.51 ± 1.79	8.00	0.00662
24	P75/G16	OJ000223_09.15, putative glyceraldehyde-3-phosphate dehydrogenase	Os04g40950	1.80 ± 0.22	4.74 ± 0.31	2.64	0.00018
25	p771/g45	Predicted OJ1791_B03.34 gene product, putative glyceraldehyde-3-phosphate dehydrogenase	Os02g38920	0.02 ± 0.003	0.07 ± 0.01	2.64	0.02800
26	-/g344	Predicted OJ1791_B03.34 gene product, putative glyceraldehyde-3-phosphate dehydrogenase	Os02g38920		0.01 ± 0.002		
27	P581/G469	Putative pyruvate kinase	Os10g42100	0.14 ± 0.01	0.39 ± 0.03	2.68	0.00069
28	P167/G53	NADP-malic enzyme	Os05g09440	0.70 ± 0.04	2.91 ± 0.15	4.14	0.00172
29	P567/G527	NADP-malic enzyme	Os05g09440	0.15 ± 0.03	0.35 ± 0.25	2.36	0.00134
30	P50/G8	Malate dehydrogenase	Os01g46070	2.64 ± 0.35	8.04 ± 0.76	3.05	0.00155
31	P60/G10	OSJNBa0044K18.22, containing domain pfam00180, putative isocitrate/isopropylmalate dehydrogenase	Os04g40310	2.27 ± 0.15	7.74 ± 0.37	3.41	0.00016
32	P58/G14	OSJNBa0044K18.22, containing domain pfam00180, putative isocitrate/isopropylmalate dehydrogenase	Os04g40310	2.32 ± 0.19	6.07 ± 0.36	2.61	0.00051
33	P302/G127	NADP-specific isocitrate dehydrogenase	Os01g46610	0.33 ± 0.05	1.33 ± 0.09	3.98	0.00043
34	p784/g75	Putative succinyl-CoA ligase α subunit	Os07g38970	0.02 ± 0.01	0.05 ± 0.004	2.14	0.01471
35	P372/G279	Putative xylulose kinase	Os07g44660	0.25 ± 0.03	0.61 ± 0.04	2.42	0.00023
36	P192/G38	ATP synthase F_0 subunit 1	Mitochondria	0.58 ± 0.05	3.67 ± 0.65	6.29	0.01431
37	-/G48	Putative ATP synthase	Os02g03860		3.13 ± 0.38		
38	P331/G211	Putative NADPH-thioredoxin reductase	Os02g48290	0.29 ± 0.02	0.80 ± 0.07	2.79	0.00654
39	P476/G190	OSJNBa0067K08.22, putative cytochrome oxidase c subunit 6b	Os07g42910	0.18 ± 0.03	0.89 ± 0.09	4.83	0.00099
40	-/G189	Putative soluble inorganic pyrophosphatase	Os10g26600		0.90 ± 0.15		
41	-/G42	Putative soluble inorganic pyrophosphatase	Os10g26600		3.40 ± 0.41		
42	-/G394	Putative soluble inorganic pyrophosphatase	Os10g26600		0.46 ± 0.08		
43	-/G39	Putative soluble inorganic pyrophosphatase	Os10g26600		3.58 ± 0.21		
44	-/G103	Putative soluble inorganic pyrophosphatase	Os05g36260		1.58 ± 0.12		

TABLE I—*continued*

IN[a]	Spot no.[b]	Protein name	Chr.[c] locus	NPV[d]	NGV[d]	NCT[e]	*p* value[f]
Protein metabolism							
45	P361/G247	eIF4A	Os06g48750	0.26 ± 0.03	0.70 ± 0.06	2.69	0.00137
46	P528/G75	Unknown protein, containing the eIF4G domain smart00544	Os05g05450	0.16 ± 0.02	2.10 ± 0.12	12.88	0.00123
47	P108/G24	Putative dnaK-type molecular chaperone BiP	Os02g02410	1.22 ± 0.15	4.11 ± 0.45	3.37	0.00787
48	P110/G44	Heat shock protein 70	Os11g47760	1.10 ± 0.09	3.33 ± 0.14	3.03	0.00018
49	P677/G177	Oligopeptidase A-like, peptidase M3 family, containing the peptidase M3 domain pfam01432	Os02g58340	0.11 ± 0.01	0.96 ± 0.08	8.61	0.00293
50	-/g218	OSJNBa0065O17.12, containing the domain pfam00082, peptidase S8	Os04g47150		0.13 ± 0.02		
51	-/g446	Putative subtilisin-like proteinase	Os06g40700		0.06 ± 0.01		
52	P570/G255	Putative leucine aminopeptidase, containing peptidase M17 domain pfam00883	Os02g55140	0.15 ± 0.02	0.47 ± 0.08	3.20	0.02153
53	P142/G33	20 S proteasome subunit α1	Os03g08280	0.87 ± 0.10	3.74 ± 0.42	4.31	0.00719
54	-/G113	Predicted OJ1626_B09.4 gene product, 20 S proteasome subunit α2	Os02g42320		1.47 ± 0.29		
55	P558/G373	20 S proteasome subunit α7	Os01g59600	0.15 ± 0.02	0.48 ± 0.06	3.14	0.00256
56	P399/G201	20 S proteasome subunit β1	Os06g04800	0.23 ± 0.03	0.84 ± 0.02	3.58	0.00001
57	P277/G144	20 S proteasome β subunit 2	Os05g09490	0.37 ± 0.05	1.24 ± 0.20	3.31	0.01851
58	P383/G324	20 S proteasome subunit β6	Os09g32800	0.24 ± 0.03	0.54 ± 0.06	2.19	0.00432
Amino acid metabolism							
59	P373/G140	Putative dehydrogenase, containing aspartate-semialdehyde dehydrogenase domain COG0136	Os03g55280	0.25 ± 0.03	2.90 ± 0.18	11.45	0.00153
60	P379/G68	OSJNBb0004G23.10 containing pfam00491 domain, putative arginase	Os04g01590	0.25 ± 0.02	2.27 ± 0.33	9.19	0.00882
61	P711/G240	Cysteine synthase	Os12g42980	0.11 ± 0.01	0.72 ± 0.04	6.85	0.01772
62	P738/G470	Acireductone dioxygenase 2, containing ARD domain pfam03079	Os03g06620	0.10 ± 0.02	0.38 ± 0.04	3.94	0.01889
63	P699/G608	OSJNBa0064H22.2, putative glutamate decarboxylase and related PLP-dependent proteins	Os04g37460	0.11 ± 0.02	0.31 ± 0.01	2.85	0.00586
64	P249/G153	Glutathione reductase	Os02g56850	0.43 ± 0.06	1.17 ± 0.18	2.75	0.02011
Cytoskeleton dynamics							
65	-/G163	Actin	Os03g61970		1.06 ± 0.26		
66	-/G54	Actin	Os11g06390		1.17 ± 0.23		
67	-/G321	Actin	Os11g06390		0.54 ± 0.12		
68	-/G121	Actin	Os03g50890		1.37 ± 0.22		
69	-/G122	Actin	Os03g50890		1.37 ± 0.21		
70	-/G184	Actin	Os03g50890		0.91 ± 0.15		
71	P127/G31	Actin	Os03g50890	0.96 ± 0.21	3.83 ± 0.54	3.98	0.00332
72	P507/G173	α-Tubulin	Os03g51600	0.17 ± 0.04	0.99 ± 0.15	5.71	0.01117
Signal transduction							
73	-/G95	GDP dissociation inhibitor protein OsGDI1	Os05g34540		1.67 ± 0.30		
74	P212/G110	G protein β subunit-like	Os01g49290	0.54 ± 0.12	1.47 ± 0.07	2.72	0.00135
Ion transport							
75	P492/G248	Putative voltage-dependent anion channel protein	Os01g51770	0.18 ± 0.03	0.70 ± 0.05	4.00	0.0001
76	P777/G287	Putative voltage-dependent anion channel protein	Os01g51770	0.09 ± 0.01	0.60 ± 0.04	6.70	0.00241
77	P654/G597	Putative voltage-dependent anion channel protein	Os01g51770	0.12 ± 0.02	0.31 ± 0.04	2.61	0.00509
Stress response							
78	-/G210	Putative ascorbate peroxidase	Os03g17690		0.84 ± 0.07		
79	P154/G73	Manganese-superoxide dismutase	Os05g25850	0.76 ± 0.08	3.58 ± 0.42	4.70	0.00747
80	P460/G286	Putative legumin-like protein	Os01g74480	0.19 ± 0.03	0.60 ± 0.07	3.10	0.00296
81	P254/G77	Putative legumin	Os05g02520	0.42 ± 0.07	2.04 ± 0.31	4.87	0.01219
82	-/G417	Putative legumin	Os05g02520		0.43 ± 0.05		
Transcriptional regulation-related							
83	P864/G856	Putative leucine-rich protein	Os02g38040	0.07 ± 0.01	0.19 ± 0.01	2.84	0.00033
Nucleotide acid metabolism							
84	P402/G322	Unknown protein, putative ADP-ribose pyrophosphatase	Os05g02640	0.23 ± 0.03	0.54 ± 0.03	2.34	0.00036
85	P511/G155	Putative deoxycytidine deaminase, containing nucleoside deaminase domain cd01285	Os03g61810	0.17 ± 0.02	1.15 ± 0.09	6.82	0.00297

TABLE I—*continued*

IN[a]	Spot no.[b]	Protein name	Chr.[c] locus	NPV[d]	NGV[d]	NCT[e]	*p* value[f]
86	-/g433	Putative adenylyl cyclase-associated protein	Os08g34780		0.01 ± 0.001		
Miscellaneous							
87	-/G265	Putative UDP-glucose:flavonoid 7-*O*-glucosyltransferase	Os01g08090		0.65 ± 0.08		
88	P582/G249	Putative flavoprotein α-subunit, having alternative splicing products	Os03g61920	0.14 ± 0.01	0.70 ± 0.09	4.86	0.00811
89	P327/G145	Putative elicitor-inducible protein EIG-J7,containing lipoxygenase homology 2 (β barrel) domain smart00308	Os02g51710	0.29 ± 0.04	1.21 ± 0.19	4.13	0.01406
Unknown proteins							
90	P368/G284	Unknown protein	Os08g12520	0.25 ± 0.03	0.60 ± 0.05	2.35	0.00228
91	P556/G484	Expressed protein, containing Wali7 domain cd01910	Os03g58170	0.15 ± 0.03	0.38 ± 0.02	2.47	0.00069
92	P572/G264	B1189A09.36, containing DUF410 domain pfam04190	Os01g07100	0.15 ± 0.01	0.65 ± 0.05	4.42	0.00306
93	P703/G317	Unknown protein	Os02g41910	0.11 ± 0.02	0.55 ± 0.07	5.14	0.0086
94	P773/G196	Unknown protein	Os05g01050	0.09 ± 0.01	0.87 ± 0.08	9.65	0.00326

[a] Number of identities.

[b] Prefix "P" or "p" and "G" or "g" represent MPG and GPG sample, respectively. "P*n*/-" or "p*n*/-" and "-/G*n*" or "-/g*n*") indicate MPG- and GPG-specific spots, respectively. The number of spots is identical to that in Figs. 4 and 5.

[c] Chromosome.

[d] NPV and NGV indicate normalized RV values of these differentially expressed spots identified on MPG and GPG gels, respectively. Results shown here are mean ± S.D. of triplicate biological repeats performed on MPGs and GPGs.

[e] NCT column indicates changed times in the normalized RV values of differentially expressed protein spots upon germination.

[f] *t* test indicates significant difference in expression of these proteins between MPGs and GPGs (*p* value <0.05).

changes in levels of four marker proteins in the MPGs and GPGs (Fig. 2). β-1,4-Xylanase, a main pollen coat protein of maize (20) and rice (14), which is deposited into the pollen coat from tapetal cells of anthers (20) and whose transcripts are absent in MPGs and GPGs (Fig. 3), was present at a high level in MPGs but barely detectable in GPGs (Fig. 2). This finding is consistent with the role of β-1,4-xylanase in hydrolyzing the cell wall of stigmas by being released onto the stigma from geminating pollen (20). Catalase, a peroxisomal protein (21), and OsRad21-3 (25), a nucleus-localizing protein, showed no change in level, but plasma membrane ATPase was at a high level in GPGs. Up-regulation of plasma membrane ATPase (22) was in accordance with active proton exchange across the plasma membrane required for polar tube growth (11).

Furthermore we analyzed the accumulation of the transcripts of six marker proteins in anthers, MPGs, and GPGs by RT-PCR. As shown in Fig. 3, the transcript encoding β-1,4-xylanase was present in anthers but undetectable in MPGs and GPGs. This result is consistent with previous data that pollen β-1,4-xylanase is from sporophytic anther tissues (20) and also indicated that at the molecular level the MPGs and GPGs we used were not contaminated with other tissues. However, transcripts encoding other marker proteins were in high abundance in GPGs and MPGs as compared with anthers (Fig. 3). This finding suggests that the germinated pollen has a normal level of transcripts. All these data indicate that the *in vitro* germinated pollen should have a set of metabolism reactions similar to those in *in vivo* germinated pollen. Thus, these GPGs were further used for proteomics identification of differentially expressed proteins.

Protein Expression Profiles of GPGs—To identify differentially expressed proteins associated with germination and early tube growth, we analyzed protein expression profiles of MPGs and GPGs by 2-DE and CBB staining. Originally we separated the two distinct protein samples by 2-DE on pH 3–10 gel strips, performed in at least triplicate biological repeats to ensure the reproducibility of protein patterns. The experiments revealed 559 ± 10 spots ($n = 3$) for MPGs (Fig. 4*A*) and 544 ± 11 ($n = 3$) for GPGs (Fig. 4*B*). Most of the spots were distributed around pH 4–7 with 413 ± 7 for MPGs and 404 ± 11 for GPGs and only about 26% around pH 7–10 (146 ± 6 for MPGs and 140 ± 5 for GPGs). The protein spots around pH 7–10 were well separated with ≧90% well matched spots in triplicate biological repeats of MPGs and GPGs. Therefore we identified differentially expressed proteins from these well matched spots in the pH 7–10 range of the two distinct gels by comparison and quantification. Statistical analysis revealed five RV-increased, 10 RV-reduced (*p* value <0.05), eight MPG-specific, and 17 GPG-specific spots (Fig. 4 and Tables I and II).

Because the first separation on pH 3–10 gel strips showed that most proteins from MPGs and GPGs were distributed around pH 4–7, to better resolve these proteins, we further separated them by 2-DE with pH 4–7 gel strips. The second separation revealed better resolution with 1004 ± 22 ($n = 3$) spots for MPGs (Fig. 5*A*) and 1030 ± 45 for GPGs ($n = 3$) (Fig. 5*B*). Among these spots, 813 on MPG and 824 on GPG gels

appeared to be well matched in triplicate biological repeats. When the well matched spots from the two distinct protein profiles were compared and quantified, 63 protein spots showed at least a 2-fold increase in level and 42 spots showed at least a 2-fold decrease in GPG gels (p value <0.05), whereas 15 spots in MPG gels were not detected in GPG gels, and 26 new spots appeared in GPG gels (Fig. 5 and Tables I and II).

Taken together, our analyses detected 66 development stage-specific protein spots and 120 spots with a changed level of expression in the MPG and GPG protein profiles (186 in total) (Figs. 4 and 5 with close-up views for eight in Supplemental Fig.S1 as examples). Finally our MS analyses led to the identification of 160 protein spots, 126 identified by PMFs on MALDI-TOF MS (Fig. 6*A*), and 34 identified by amino acid sequences of peptides on ESI Q-TOF MS/MS (Fig. 6, *B* and *C*) (Supplemental Table S1). The 160 identities represent 120 unique proteins (Unipros) (Tables I and II).

Characteristics of the Differentially Expressed Proteins—These RV-increased and newly appearing protein spots (Table I) can be considered up-regulated in pollen germination and tube growth. But numerous studies have revealed that some or all proteins in the pollen coat, a specialized layer outside the wall, and several pollen wall-associated species, either from pollen itself or from sporophytic tissues of anthers, are releasable during pollen germination (14, 20); and after pollination and germination on the stigma, the germinating pollen will release some interior proteins for interaction with the stigma to facilitate tube growth in pistils. Thus, the RV-reduced proteins or those that have disappeared in the germinated pollen may result from real down-regulated expression and/or release of coat/wall-associated and pollen-interior proteins into the germination medium.

In this study, we tried to detect these released proteins from germination medium but were unsuccessful mainly because of the high concentration of sucrose and polyethylene glycol in the germination medium interfering with the separation by one-dimensional electrophoresis, 2-DE, or liquid chromatography (data not shown). However, 21 of the 66 identified RV-reduced/disappearing protein spots (Table II), such as β-1,4-xylanase, pollen allergens, β-expansin, polygalacturonase, profilin A, enolase, and ascorbate peroxidase, have been detected in coat/wall-associated and pollen-released protein fractions of mature rice (14) and maize pollen (20). Further analyses revealed that 1) most of the identified RV-reduced/disappearing proteins, such as members of the class III peroxidase family, polygalacturonase, and disulfide isomerase, have a potential extracellular targeting signal peptide in their N termini (Table II), and 2) 24 have been documented to be secreted/released extracellular proteins and/or cell surface/wall-associated proteins in other organisms, for example 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (26), fructose-1,6-bisphosphate aldolase (27), and triose-phosphate isomerase (28). Thus, we propose that 51 of the 66 RV-reduced/disappearing identities (39 Unipros) were probably released, and the other 15 (14 Unipros) may really be down-regulated (Table II).

Functional Categories of These Differentially Expressed Identities—Among the 160 differentially expressed identities, 128 (80%) have been deposited in the current database as putative functional proteins. We further examined their annotated function by domain searching and similarity comparison. The remaining 32 (20%) were deposited in the database as unknown or hypothetical proteins. Domain searches and similarity analyses revealed that 20 of the unknown proteins contained conserved entire domains associated with known activities and/or showed high sequence similarity with known functional proteins in the database. The other 12, with no known conserved domains or sequence similarity with known functional proteins, may represent a group of novel proteins.

From the above analyses, in combination with metabolic and functional features of pollen, we grouped all the identities into 13 major categories (Fig. 7*A*). An impressive 74% of these identities were implicated in five functional groups, including carbohydrate/energy metabolism (24%), wall remodeling and metabolism (24%), protein metabolism (11%), cytoskeleton dynamics (8%), and stress response (7%) (Fig. 7*A*). Analysis of their relative expression level in each category revealed an identical trend (Fig. 7*B*); these cellular/metabolic process-related proteins were overrepresented either in number or expression level of the identified differentially expressed proteins, suggesting the functional importance of these processes in pollen tube growth.

Differentially Expressed Proteins Are Associated Preferentially with Carbohydrate and Energy Metabolism—Starch is preferentially stored in rice pollen and metabolized upon germination to supply carbon skeletons and energy for the growing pollen tube. Thus, mature rice pollen has presynthesized these enzymes for carbohydrate/energy metabolism (14). Our results show that of these differentially expressed identities those implicated in the functional category of carbohydrate and energy metabolism as well as wall metabolism were the highest represented. Furthermore of the function group-related 38 identities, 27 were up-regulated in the germinated pollen, including those essential for starch degradation and sucrose synthesis (α-1,4-glucan phosphorylase H and sucrose-6^F-phosphate phosphohydrolase), glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway (Table I). These up-regulated proteins also included ATP generation-related proteins such as ATP synthase F_O subunit 1, ATP synthase, NADPH-thioredoxin reductase, and cytochrome oxidase c subunit 6b (Table I), and basic energy supply-related soluble inorganic pyrophosphatase (five isoforms: spots -/G189, -/G42, -/G394, -/G39, and -/G103) (Table I). Therefore, these data demonstrate that active carbohydrate/energy metabolism is a basis of fast pollen tube growth.

Unexpectedly 10 carbohydrate metabolism-related identities were detected as RV-reduced/disappearing protein spots in the germinated pollen (Table II). They represented eight

TABLE II

Proteins identified in rice by 2-DE and MS whose RV was reduced on pollen germination

TPA, third party annotation.

IN[a]	Spot no.[b]	Protein name	Chr.[c] locus	C[d]	S[e]	SCL[f]	Ref.[g]	NPV[h]	NGV[h]	NCT[i]	*p* value[j]
Wall remodeling and metabolism											
1	p952/g433	Putative polygalacturonase	Os06g35320	R	S	Vac (0.90); Out (0.77)	64	0.01 ± 0.01	0.001 ± 0.0003	6.84	0.00206
2	p565/g148	Putative polygalacturonase	Os06g35320	R	S	Vac (0.90); Out (0.77)	64	0.21 ± 0.02	0.02 ± 0.003	10.50	0.0021
3	p621/-	Putative polygalacturonase	Os06g35320	R	S	Vac (0.90); Out (0.77)	64	0.08 ± 0.01			
4	p587/g237	Putative 1,4-β -xylanase	Os10g21110	R		Cyt (0.45); Per (0.42)	20	0.14 ± 0.02	0.01 ± 0.0015	10.53	0.00608
5	P18/G86	Putative group 3 pollen allergen	Os06g44470	R	S	Out (0.82); Vac (0.38)	14	6.74 ± 0.90	1.79 ± 0.20	3.77	0.01141
6	P3/G17	Putative group 3 pollen allergen	Os06g45180	R	S	Vac (0.90); Out (0.82)	14	14.40 ± 1.70	4.60 ± 0.30	3.13	0.01019
7	P11/G34	OSJNBa0050F15.10, containing pfam01357 domain, putative pollen allergen 1	Os04g25190	R	S	Out (0.82); Vac (0.37)	36	8.96 ± 0.64	3.74 ± 0.41	2.40	0.00133
8	P17/G116	OSJNBa0050F15.8, putative pollen allergen 1	Os04g25160	R	S	Out (0.74); Vac (0.30)	14	14.40 ± 1.55	4.60 ± 0.51	3.13	0.00917
9	P87/-	Putative pollen allergen Phl p 11	Os06g36240	R	S	Out (0.82); ER-m (0.10)	35	1.54 ± 0.17			
10	P150/-	Putative pollen-specific protein C13 precursor, containing pollen Ole e I function domain pfam 01190	Os09g39950	R	S	Pm (0.69); ER-m (0.64)	35	0.79 ± 0.09			
11	P47/G269	Putative pollen-specific protein C13 precursor, containing pollen Ole e I function domain pfam 01190	Os09g39950	R	S	Pm (0.69); ER-m (0.64)	35	2.83 ± 0.21	0.64 ± 0.05	4.43	0.00318
12	P29/-	β -Expansin OsEXPB13	Os03g01650	R	S	Vac (0.90); Out (0.82)	65	4.72 ± 0.58			
13	P9/G22	β -Expansin OsEXPB13	Os03g01650	R	S	Vac (0.90); Out (0.82)	14	9.61 ± 1.10	4.15 ± 0.31	2.31	0.0143
14	p554/g82	β -Expansin	Os10g40090	R	S	Out (0.82); Pm (0.19)		0.31 ± 0.05	0.05 ± 0.01	6.68	0.01396
15	P15/G273	TPA: class III peroxidase 31	Os02g50770	R	S	Pm (0.46); ER-m (0.10)	66	7.49 ± 0.80	0.63 ± 0.10	11.97	0.04544
16	P1/G133	TPA: class III peroxidase 36	Os03g05770	R	S	Vac (0.90); Out (0.82)		16.87 ± 1.83	1.31 ± 0.15	12.91	0.00461
17	p594/g126	TPA: class III peroxidase 36	Os03g05770	R	S	Vac (0.90); Out (0.82)		0.13 ± 0.01	0.03 ± 0.01	4.21	0.00034
18	P52/G335	Putative dirigent-like protein	Os03g59440	R	S	Out (0.82); ER-m (0.10)		2.46 ± 0.15	0.53 ± 0.04	4.66	0.00211
19	P190/-	Apospory-associated protein C-like	Os09g15820	R	S	ER-m (0.55); Out (0.10)	67	0.59 ± 0.05			
20	p616/-	Expressed protein, similar to cell wall protein FLO11p (Os11g11730)	Os11g11710	R	S	Out (0.82); Vac (0.32)		0.09 ± 0.01			
21	p583/-	Expressed protein, similar to cell wall protein FLO11p (Os11g11730)	Os11g11710	R	S	Out (0.82); Vac (0.32)		0.16 ± 0.02			
22	p592/-	Expressed protein, similar to cell wall protein FLO11p (Os11g11730)	Os11g11710	R	S	Out (0.82); Vac (0.32)		0.13 ± 0.02			
23	P41/G111	UDP-glucose pyrophos-phorylase	Os02g02560	R		Cyt (0.45); Per (0.30)	14	3.30 ± 0.13	1.48 ± 0.12	2.23	0.00005

TABLE II—*continued*

IN[a]	Spot no.[b]	Protein name	Chr.[c] locus	C[d]	S[e]	SCL[f]	Ref.[g]	NPV[h]	NGV[h]	NCT[i]	*p* value[j]
Carbohydrate and energy metabolism											
24	p561/g96	Cytoplasmic aldolase, putative fructose-1,6-bisphosphate aldolase	Os05g33380	R		Cyt (0.60); Mit (0.10)	27	0.24 ± 0.01	0.04 ± 0.005	6.36	2.40E06
25	P149/G833	Putative fructokinase I	Os01g66940	R		Cyt (0.45); Per (0.35)	14	0.79 ± 0.11	0.21 ± 0.02	3.85	0.01146
26	P36/-	Putative triose-phosphate isomerase	Os01g05490	R		Cyt (0.65); Mit (0.10)	28	3.22 ± 0.23			
27	P217/G710	Putative phosphoglycerate kinase	Os02g07260	R		Nuc (0.76); Per (0.30)	29	0.52 ± 0.07	0.25 ± 0.02	2.04	0.02449
28	P79/G243	Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	Os01g60150	R		Cyt (0.45); Per (0.43)	26	1.70 ± 0.21	0.71 ± 0.09	2.39	0.05137
29	P42/G167	Putative 2,3-bisphosphoglycerate-independent phosphoglycerate mutase	0s05g40420	R		Cyt (0.45); Per (0.43)	26	3.27 ± 0.11	1.03 ± 0.12	3.16	0.00002
30	P23/G101	Enolase	Os10g08550	R		ER-m (0.60); Mit-im (0.41)	30	6.17 ± 0.45	1.60 ± 0.21	3.86	0.00055
31	P24/G106	Enolase	Os10g08550	R		ER-m (0.60); Mit-im (0.41)	30	5.49 ± 0.53	1.56 ± 0.17	3.52	0.00675
32	P62/-	Putative 6-phospho-gluconolactonase	Os09g35970	R		Per (0.43); Nuc (0.30)	14	2.10 ± 0.31			
33	P107/G468	β -Phosphoglucomutase-like protein	Os09g24230	D		Pm (0.44); Mit-im (0.38)		1.25 ± 0.08	0.39 ± 0.04	3.23	0.00109
34	p696/-	Putative pyrophosphate-dependent phosphofructo-1-kinase	Os06g05860	D		Chl-m (0.92); Chl-tm (0.71)		0.04 ± 0.006			
Protein metabolism											
35	P120/-	Putative disulfide isomerase	Os05g06430	R	S	Vac (0.90); Out (0.82)	68	1.02 ± 0.12			
36	P102/G325	Protein-disulfide isomerase	Os11g09280	R	S	ER (0.80); Per (0.50)	68	1.29 ± 0.09	0.54 ± 0.04	2.39	0.00107
37	P237/G785	21-kDa polypeptide, putative TCTP	Os11g43900	D		Per (0.75), Mit-ms (0.10)		0.46 ± 0.05	0.23 ± 0.03	2.04	0.00218
Cytoskeleton dynamics											
38	P4/G21	Profilin A	Os10g17680	R		Per (0.58); Cyt (0.45)	20	13.76 ± 2.20	4.28 ± 0.60	3.22	0.01888
39	P218/G870	β -Tubulin	Os06g46000	D		Cyt (0.45); Mit-ms (0.10)		0.52 ± 0.11	0.19 ± 0.03	2.72	0.03397
40	P219/G817	β-Tubulin	Os05g34170	D		Cyt (0.45); Per (0.11)		0.52 ± 0.11	0.21 ± 0.03	2.44	0.0426
41	P61/G160	β -Tubulin	Os03g45920	D		Cyt (0.45); Per (0.14)		2.21 ± 0.31	1.09 ± 0.17	2.03	0.01089
42	P67/G204	α -Tubulin	Os07g38730	D		Cyt (0.45); Per (0.31)		1.96 ± 0.32	0.83 ± 0.07	2.36	0.02694
Signal transduction											
43	P207/G914	Putative Rho GDP dissociation inhibitor	Os01g68540	R		Cyt (0.45); Per (0.30)	14	0.55 ± 0.07	0.17 ± 0.04	3.22	0.004607
44	P27/G90	Putative calreticulin	Os07g14270	R	S	Vac (0.90); Out (0.82)	69	4.76 ± 0.72	1.74 ± 0.33	2.73	0.00717
45	P72/G537	Putative calreticulin	Os03g61670	R	S	ER-l (0.91); Out (0.82)	69	1.84 ± 0.46	0.34 ± 0.13	5.33	0.03208
46	P96/G430	Zinc finger and C2 domain protein-like	Os07g01780	R		Mit-ms (0.47); Cyt (0.45)	14	1.40 ± 0.25	0.42 ± 0.17	3.37	0.011267
Ion transport											
47	p690/-	VDAC	Os05g45950	D		Per (0.50); Cyt (0.45)		0.04 ± 0.01			

TABLE II—*continued*

IN[a]	Spot no.[b]	Protein name	Chr.[c] locus	C[d]	S[e]	SCL[f]	Ref.[g]	NPV[h]	NGV[h]	NCT[i]	*p* value[j]
48	P182/-	Putative vacuolar proton-ATPase	Os06g45120	D		Cyt (0.45); Per (0.39)		0.64 ± 0.08			
49	P34/G85	Putative vacuolar proton-ATPase	Os06g45120	D		Cyt (0.45); Per (0.39)		4.02 ± 0.33	1.79 ± 0.21	2.24	0.00227
Vesicle trafficking											
50	P179/-	Putative lysophospholipase 2	Os01g07960	R		Cyt (0.45); Per (0.34)	14	0.66 ± 0.08			
Stress response											
51	P38/G97	L-Ascorbate peroxidase	Os07g49400	R		Per (0.51); Cyt (0.45)	14	3.49 ± 0.37	1.63 ± 0.11	2.14	0.014
52	P64/-	Putative ascorbate peroxidase	Os03g17690	R		Per (0.47); Cyt (0.45)	14	2.09 ± 0.31			
53	P13/-	Putative ascorbate peroxidase	Os03g17690	R		Per (0.47); Cyt (0.45)	14	7.78 ± 0.92			
54	P111/G336	Dehydroascorbate reductase	Os05g02530	R		Cyt (0.45); Per (0.30)	14	1.09 ± 0.11	0.53 ± 0.06	2.06	0.00487
55	P20/G58	Putative legumin	Os05g02520	R		Per (0.55); Chl (0.20)	14	6.31 ± 0.65	2.80 ± 0.23	2.26	0.01296
Nucleotide acid metabolism											
56	P143/G489	Adenosine kinase-like protein	Os02g41590	D		Cyt (0.65); Mit-ms (0.10)		0.85 ± 0.09	0.37 ± 0.01	2.28	0.01053
57	P193/G731	Putative dihydropyrimidine dehydrogenase	Os02g50350	D		Mit-ms (0.59); Mit-im (0.30)		0.58 ± 0.05	0.24 ± 0.03	2.36	0.00189
Unknown proteins											
58	P14/-	Unknown protein	Os08g12520	R	S	Out (0.82); ER-l (0.10)	14	7.34 ± 0.91			
59	p549/g23	Unknown protein	Os09g24120	R	S	Out (0.82); Vac (0.44)	14	0.38 ± 0.04	0.09 ± 0.01	4.03	0.00795
60	613/200	Unknown protein	Os09g24120	R	S	Out (0.82); Vac (0.44)	14	0.09 ± 0.005	0.02 ± 0.012	5.17	0.00222
61	P8/G43	Unknown protein	Os09g24130	R	S	Out (0.85); Vac (0.82)	14	10.49 ± 2.10	3.34 ± 0.54	3.14	0.02976
62	p652/g308	Unknown protein	Os03g10510	R	S	Out (0.79); Vac (0.44)		0.06 ± 0.004	0.01 ± 0.002	6.49	0.00036
63	p619/g199	Hypothetical protein	Os11g08860	R	S	Out (0.82); ER-l (0.10)		0.08 ± 0.006	0.02 ± 0.008	3.99	0.00063
64	P65/G169	Unknown protein	Os01g13440	D	S	Pm (0.46); ER-m (0.10)		2.02 ± 0.13	1.01 ± 0.25	2.00	0.00858
65	p987/-	Unknown protein	Os01g64900	D		Cyt (0.65); Chl-s (0.20)		0.03 ± 0.004			
66	P59/G347	Hypothetical protein	Os03g27270	D		Per (0.30); Nuc (0.30)		2.27 ± 0.35	0.51 ± 0.08	4.42	0.0136

[a] Number of identities.
[b] See Footnote b of Table I.
[c] Chromosome.
[d] Changes in expression level of these proteins upon germination. R, probable released proteins; D, down-regulated proteins.
[e] Signal peptide predicted by SignalP program. S, signal peptide.
[f] The protein subcellular localization predicted by PSORT program. The top two probabilities and their scores for each protein are shown. Cyt, cytoplasm; Per, peroxisome; Pm, plasma membrane; ER; endoplasmic reticulum; ER-m, ER membrane; Vac, vacuole; Out, outside; Mit, mitochondria; Mit-im, mitochondria inner membrane; Nuc, nucleus; Chl-m, chloroplast stroma; Chl-tm, chloroplast thylakoid membrane; Chl-s, chloroplast stroma; Mit-ms, mitochondria matrix space; ER-l, endoplasmic reticulum lumen.
[g] References documenting that the proteins are in membrane/cell wall or probably released.
[h] See Footnote d in Table I.
[i] See Footnote e in Table I.
[j] *t* test indicates significantly different expression of these proteins in MPGs and GPGs (*p* value <0.05).

unique proteins, including enolase, triose-phosphate isomerase, phosphoglycerate kinase, phosphoglycerate mutase, fructose-1,6-bisphosphate aldolase, fructokinase I, β-phosphoglucomutase, and 6-phosphogluconolactonase. The first five are found in the cell wall of yeast or pathogens (26–30). Our proteomics analysis of MPGs also revealed that all but phosphoglycerate kinase were present in the pollen-released protein fraction (14). Therefore, their decrease in level or dis-

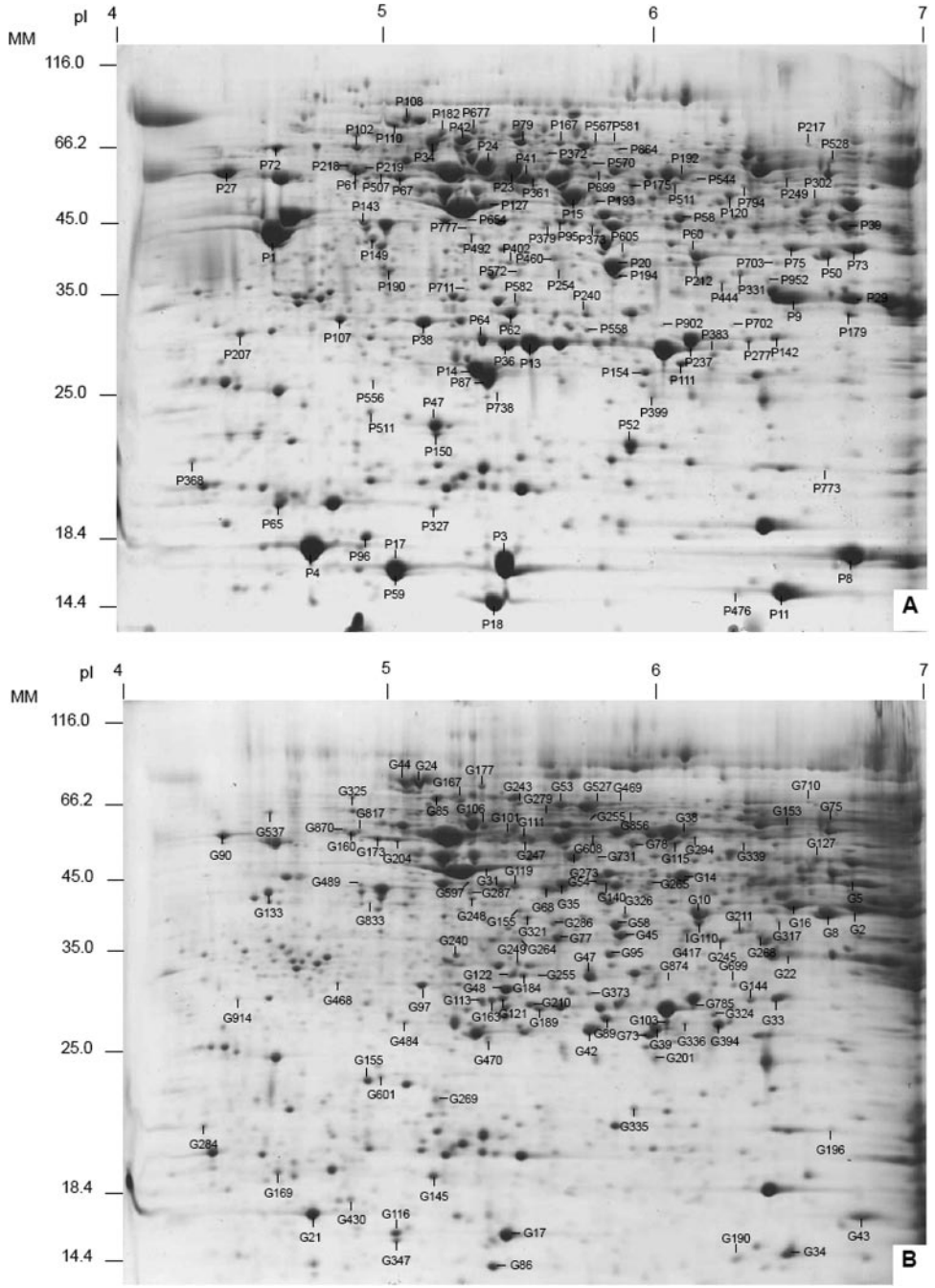
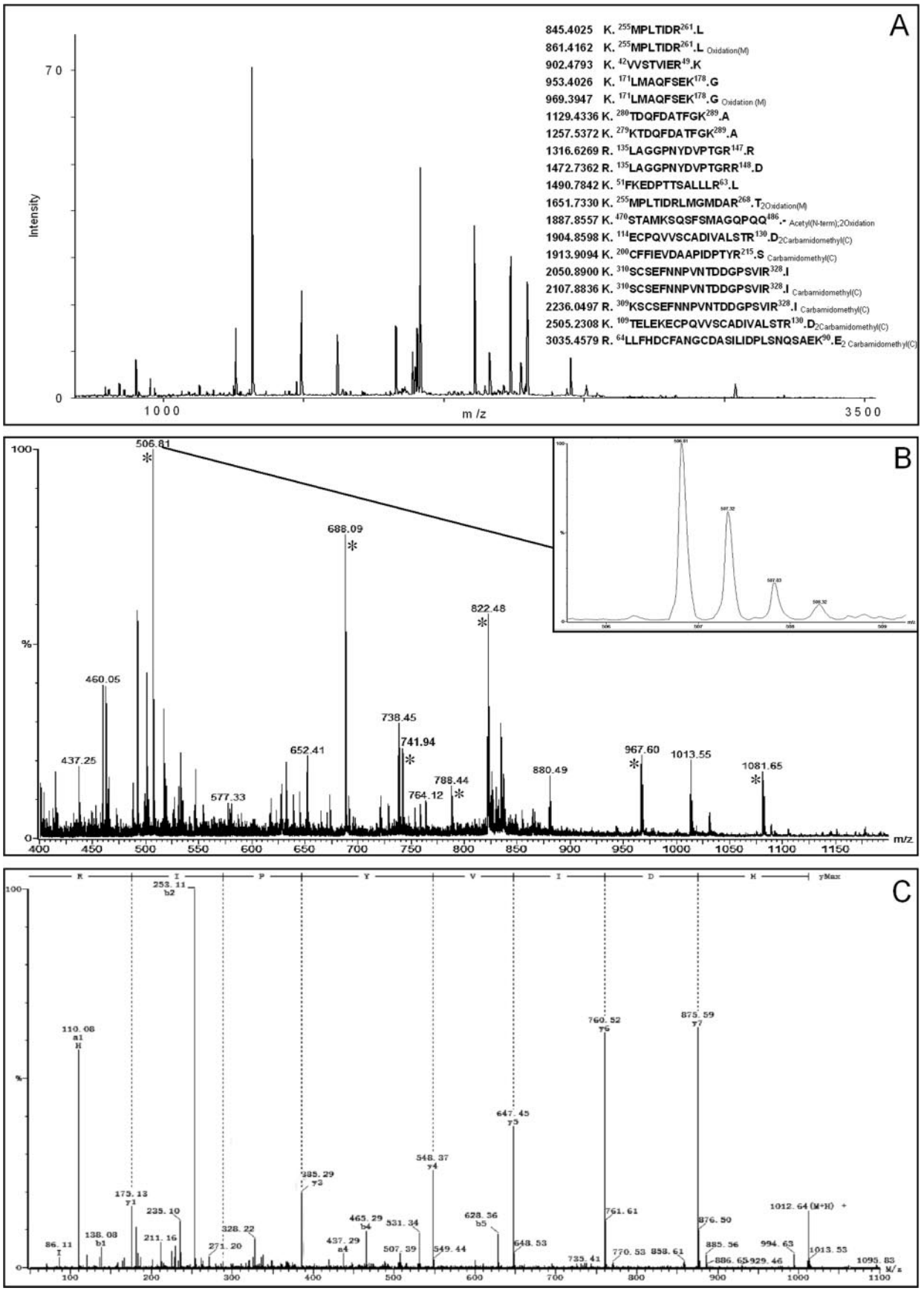


FIG. 5. **Representative 2-DE images of MPG (*A*) and GPG (*B*) proteins in the pH 4–7 range.** Proteins were separated by 2-D PAGE and stained with CBB. MM in kilodaltons and pI of proteins are indicated on the *left* and *top* of each image, respectively. An average of 1004 ± 22 MPG and 1030 ± 45 GPG protein spots were detected from three replicate repeats for of each sample. These differentially expressed protein spots after pollen germination were marked and identified. The protein spots from MPGs are numbered with a prefix "*P*," and those from GPGs are numbered with a "*G*." Numbered spots correspond to proteins listed in Tables I, II, and III and Supplemental Table S1.

appearance in the germinated pollen should result from their release. Why are these carbohydrate metabolism-related proteins releasable? One possibility is that these proteins may have a role in a related metabolism on the pollen/stigma surface or they may be deposited onto the pollen coat/wall from degraded tapetal cells (20).

Germinated Pollen Seems to Display Active Wall Dynamics Both in Wall Synthesis and Degradation—The differentially expressed wall-related proteins (39 identities, 24% total) could be divided into two distinct function groups. The first group consists of 16 up-regulated identities (Table I) seeming to function in wall synthesis and hydrolyzing/loosening with


A
845.4025 K. 255MPLTIDR261.L
861.4162 K. 255MPLTIDR261.L Oxidation(M)
902.4793 K. 42VVSTVIER49.K
953.4026 K. 171LMAQFSEK178.G
969.3947 K. 171LMAQFSEK178.G Oxidation (M)
1129.4336 K. 280TDQFDATFGK289.A
1257.5372 K. 279KTDQFDATFGK289.A
1316.6269 R. 135LAGGPNYDVPTGR147.R
1472.7362 R. 135LAGGPNYDVPTGRR148.D
1490.7842 K. 51FKEDPTTSALLLR63.L
1651.7330 K. 255MPLTIDRLMGMDAR268.T 2Oxidation(M)
1887.8557 K. 470STAMKSQSFSMAGQPQQ486.- Acetyl(N-term);2Oxidation
1904.8598 K. 114ECPQVVSCADIVALSTR130.D 2Carbamidomethyl(C)
1913.9094 K. 200CFFIEVDAAPIDPTYR215.S Carbamidomethyl(C)
2050.8900 K. 310SCSEFNNPVNTDDGPSVIR328.I
2107.8836 K. 310SCSEFNNPVNTDDGPSVIR328.I Carbamidomethyl(C)
2236.0497 R. 309KSCSEFNNPVNTDDGPSVIR328.I Carbamidomethyl(C)
2505.2308 K. 109TELEKECPQVVSCADIVALSTR130.D 2Carbamidomethyl(C)
3035.4579 R. 64LLFHDCFANGCDASILIDPLSNQSAEK90.E 2 Carbamidomethyl(C)
Intensity
m/z
B
506.81
688.09
822.48
460.05
738.45
741.94
437.25
652.41
577.33
764.12
788.44
880.49
967.60
1013.55
1081.65
%
m/z
C
K I P Y V I D K yMax
253.11 b2
110.08 a1 H
760.52 y6
875.59 y7
647.45 y5
548.37 y4
385.29 y3
173.13 y1
235.10
465.29 b4
531.34
628.36 b5
761.61
876.50
1012.64 (M+H) +
86.11 I
138.08 b1
211.16
328.22
271.20
437.29 a4
507.39
549.44
648.53
735.41
770.53
858.61
885.56
994.63
1013.53
886.65
929.46
1095.83
M/z

seven (representing five enzymes), including UDP-glucose pyrophosphorylase, UDP-glucose dehydrogenase, dTDP-4-dehydrorhamnose reductase, reversibly glycosylated polypeptide with three isoforms (spots P902/G874, P95/G35, and -/G119), and *myo*-inositol-1-phosphate synthase, involved in the interconversion and transportation of sugars required for cell wall biosynthesis. Another nine, including multifunctional class III peroxidase (POD) 79, POD 78 with two isoforms (spots P444/G245 and P702/G699), POD 31 with two isoforms (spots P605/G326 and P194/G45), polygalacturonase Os02g10300 with two isoforms (spots -/g242 and -/g281), and expansin with two sequence-related isoforms (Os10g40090, spot -/g93; Os03g01640, spot -/g398), may be responsible for wall loosening and hydrolyzing. Class III PODs have been documented to function in promoting elongation of *Arabidopsis* roots (31, 32) or physically inhibiting the elongation process by creating cell wall cross-linking (33). Polygalacturonase is a key enzyme-degrading pectin, and expansins are involved in cell elongation growth. The up-regulation of these proteins in germinated pollen suggests that they may be necessary for fast polar tube growth through regulating synthesis and wall loosening.

The second group involves 23 possibly pollen-released identities (16 Unipros) (Table II) such as polygalacturonase Os06g35320 with three isoforms (spots p952/g433, p565/g148, and p621/-), β-1,4-xylanase, ~12-kDa β-expansin-like (spots P18/G86, P3/G17, P11/G34, and P17/G116), pollen allergen Phl p 11, pollen-specific protein C13 with two isoforms (spots P150/- and P47/G269), ~35-kDa β-expansin, and β-expansin OsEXPB13 with two isoforms (spots P29/- and P9/G22). Secretory activity of polygalacturonase has been detected in pollen of many species (1, 34). β-1,4-Xylanase is one of the most abundant pollen coat proteins in maize and functions in hydrolyzing the stigma and track cell wall (20). Pollen allergen Phl p 11 contains the pollen Ole e I domain (pfam01190), whereas Ole e 1 has been localized in the pollen wall and can be released into the culture medium during *in vitro* germination (35). Several members of the β-expansin subfamily in grass pollen are proposed to facilitate pollen tube growth by loosening the cell wall in pistils (36). This group also contained one isoform of POD 31 (spot P15/G273) and two isoforms of POD 36 (spots P1/G133 and p594/g126). Most proteins of the group have been documented to have roles in hydrolyzing walls. Given that they are possibly released in the germinated pollen, these proteins may involve hydrolysis and/or loosening of walls of the stigma and tract cells in pistils.

Most of the Differentially Expressed Proteins in the Protein Metabolism Category Involve Protein Degradation—Among 17 identities implicated in protein translation, assembly, and degradation, besides disulfide isomerase and translationally controlled tumor protein (TCTP), which were decreased in level, others were increased in level in the germinated pollen (Tables I and II). Proteins eukaryotic initiation factors 4A (eIF4A) and 4G (eIF4G), involved in protein translation regulation at the level of ribosome recruitment, showed a 2.7- and 12.9-fold increase, respectively. eIF4A, a member of the eIF4F complex, has ATP-dependent RNA helicase activity. eIF4G, another member of the complex, is a large modular protein that serves as a docking site of translation-related proteins (37). TCTP is the key inhibitor of translation elongation; it inhibits guanine nucleotide dissociation from protein elongation factor 1A (EF1A) (38). Up-regulation of eIF4A and eIF4G and down-regulation of TCTP, together with up-regulation of molecular chaperones and amino acid metabolism-associated proteins (Table I), suggest greatly increased translational efficiency in growing pollen tubes; this is consistent with the notion that active protein synthesis is required for fast pollen tube growth (9).

Interestingly 10 of the 17 protein metabolism-related identities were implicated in protein degradation with an increased level in the germinated pollen. These included six subunit components of the 14-subunit 20 S core complex of the 26 S proteasome as well as peptidase M3, peptidase S8, subtilisin-like proteinase, and leucine aminopeptidase (Table I). This finding suggests the importance of protein turn-over in pollen tube growth.

Change in Level of Actins and Tubulins during Germination Was Distinct—Our analysis revealed 13 differentially expressed identities involved in cytoskeletal dynamics. All seven identities representing actins, including Os03g50890 with four isoforms, Os11g06390 with two isoforms, and Os03g61970, were up-regulated or newly appearing (Table I). Previous studies have revealed that subapical axial actin cables in pollen tubes act to target the vesicle to the tip and/or maintain proper cytoplasmic organization of the tube; thus they are essential for polar pollen tube growth (3). These results suggest that actin cables and dynamics in the growing tube may depend on new synthesis of actins.

In contrast, except for the up-regulated α-tubulin (Os03g51600) (Table I), the other identified tubulin proteins, including three β-tubulins (Os06g46000, Os05g34170, and Os03g45920) and one α-tubulin (Os07g38730), were de-

FIG. 6. **Representative MS spectra of proteins identified by MALDI-TOF MS (*A*) and nano-ESI Q-TOF MS/MS (*B* and *C*).** *A*, PMF pattern of spot p594/g126 marked in Fig. 4. The PMF generated by MALDI-TOF MS (see "Materials and Methods") was matched to the class III peroxidase 36 precursor by searching against the NCBInr protein database with 19 mass values matched among the 23 mass values searched and a sequence coverage of 34%. The matched peptides and their corresponding peaks are listed in the map. *B*, the MS pattern of the peptides from spot -/G89 marked in Fig. 5*B* that was matched to α-1,4-glucan phosphorylase H isozyme. Seven peaks with double charge observed on the spectrum (marked by *asterisk*) underwent further MS/MS, and the *inset* shows one of these peaks with double charge (*m/z* 506.81). *C*, the fragmentation double charged ion *m/z* 506.81 underwent *de novo* sequencing.

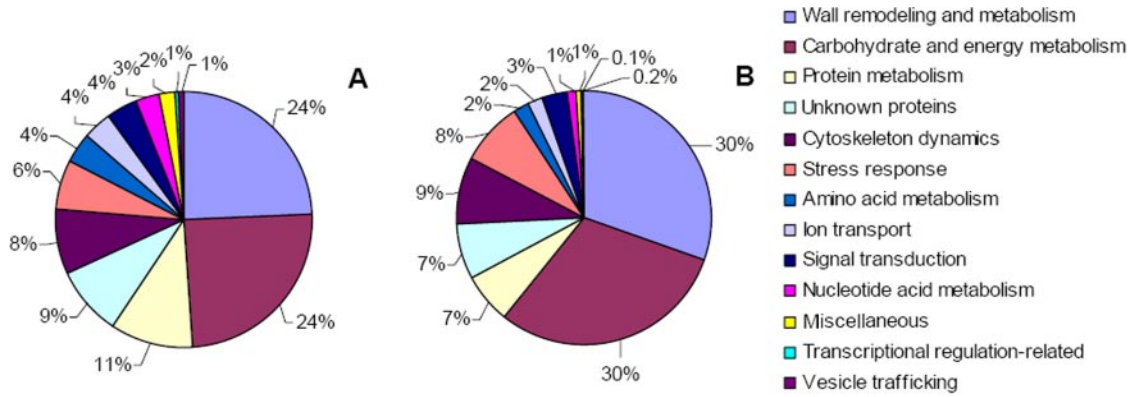


FIG. 7. **An outline of the functional classification of differentially expressed proteins.** *A*, functional distribution of all 160 identities in different categories. *B*, relative proportion of identities in each functional category (ratio of relative proportion in each category to total proportion of all identities). The proportion of identities in each functional category was the sum of the proportion of all identities in both MPGs and GPGs.

creased in level (Table II). The functions of microtubulins (MTs) in pollen tubes are not fully understood. Several studies showed that MTs had roles in transporting the male germ unit and maintaining generative cell shape (39). But depolymerization of MTs seems not to affect the initial rate of the pollen tube growth *in vitro* (1). Although evidence is lacking to explain the importance of down-regulated tubulins in polarly growing pollen tubes, their reduced level as compared with that of actin proteins suggests that the two types of cytoskeletal proteins may have different functions in tube growth.

The Presence of Multiple Isoforms—It is generally accepted that multiple isoforms result from sequence-related proteins encoded by distinct genes and/or polypeptide variants encoded by the same gene (splice variants and/or posttranslational modifications (PTMs)). Our results show that 25% (n = 30) of the differentially expressed Unipros have isoforms: six are sequence-related (actin, Os03g61970, Os11g06390, and Os03g50890; β-tubulin, Os06g46000, Os05g34170 and Os03g45920; α-tubulin, Os07g38730 and Os03g51600; voltage-dependent anion channel protein (VDAC), Os06g45210 and Os01g51770; polygalacturonase, Os02g10300 and Os06g35320; and β-expansin, Os10g40090 and Os03g01640) (Tables I and II), and the other 24 (Fig. 8 and Table III) appear to have PTM/splice variant-generated isoforms.

The 24 unique proteins appeared as 59 identities. Given that the apparent MM predicated in SDS-PAGE usually has an error deviation of about ±10% as compared with theoretical MM (40) because the apparent values are estimated according to the co-electrophoresed protein markers, we evaluated error deviations in apparent MM of these identities. Among the 59 identities, 36 appeared to have an apparent MM larger than theoretical values (>10% deviation), 12 had an apparent MM with ±10% deviation, and one had an apparent MM identical to its theoretical value (Table III). Another 10 appeared smaller than their theoretical value (≤10%) (spots -/G601, -/G89, P240/G47, -/g344, -/G121, -/G184, -/G122, P902/G874, P194/G45, P605/G326, and P702/G699, representing seven Unipros) (Table III). However, a data search in The Institute for Genomic Research (TIGR) rice database (www.tigr.org/tdb/e2k/osal/locusNameSearch.shtml) demonstrated that each of the seven genes seemed to give rise to multiple alternative splice variants, some appearing to be close to these identities in MM. Therefore, these isoforms could have resulted from splice variants rather than from proteolytic degradation. This conclusion was also evidenced by the reproducibility of all the differentially expressed identities in triplicate biological repeats and the Western blot experiments with four marker proteins, which have different subcellular localization (20–22, 25), that did not detect protein fragments possibly resulting from proteolytic degradation in the MPG and GPG protein samples (Fig. 2). Taken together, these data demonstrate the presence of these isoforms in MPGs and GPGs.

To evaluate the kinds of modifications related to the occurrence of these isoforms during pollen germination, we applied two newly developed specific staining methods (see "Detection of Glyco-/Phosphoprotein Candidates"), which are efficient to predict phosphorylated and glycosylated proteins with 2-DE gels (41), to visualize 2-DE gels of MPG and GPG proteins. A total of 14 protein spots showed positive reactions to glycoprotein detection reagent Pro-Q™ Emerald 488, four reacted to phosphoprotein detection reagent Pro-Q diamond, and one reacted to both reagents (Supplemental Fig. S2, A, B, and C, and Table III). The 19 spots represent 13 of 24 Unipros with possible PTM/splice variant-derived isoforms (Table III). In fact, 17 of the 24 protein species are glycosylated and/or phosphorylated in other organisms (Table III), and seven of the 17 have modifications identical to our results (Table III). Furthermore of the identified possible glycoproteins (Supplemental Fig. S2A and Table III), class III peroxidase has been known as a heme-containing glycoprotein (42). Polygalacturonase, pollen-specific protein C13 (Os09g39950), β-expansin OsEXPB13, and β-expansin have been well documented as pollen allergens (43–45). Previous investigations showed that

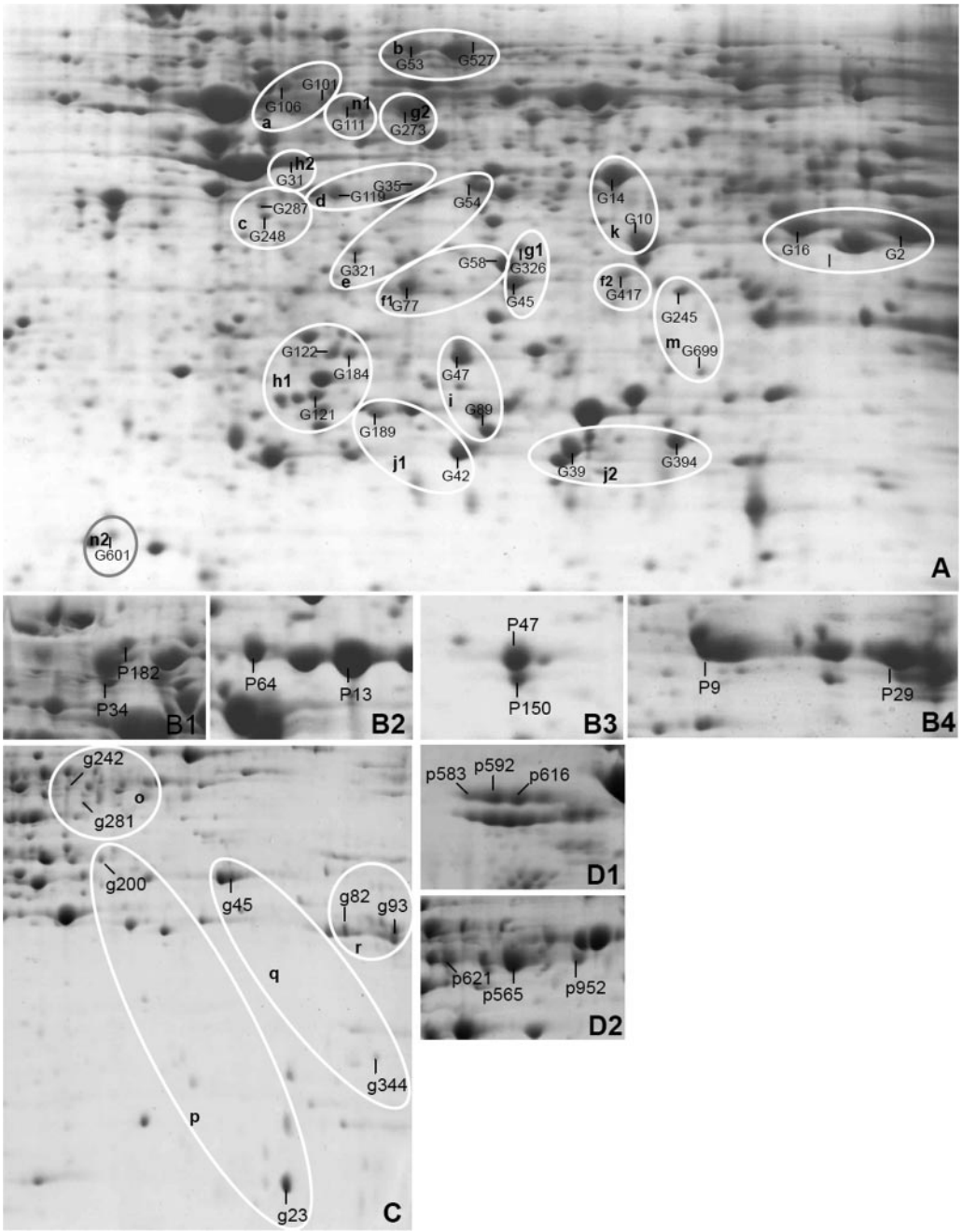


FIG. 8. **Close-up of possible isoforms generated by PTM/splice variant detected by 2-DE.** All 59 identities matched to 24 unique proteins are shown. *A*, GPG pH 4–7 gel indicating 14 protein species with isoforms. *B1–B4*, MPG pH 4–7 gel showing four protein species with isoforms. *C*, GPG pH 3–10 gel indicating four protein species with isoforms. *D1* and *D2*, MPG pH 3–10 gel indicating two protein species with isoforms. In *A*, *a*, enolase; *b*, NADP-malic enzyme; *c*, putative voltage-dependent anion channel protein; *d*, reversibly glycosylated polypeptide; *e*, actin (Os11g06390); *f1* and *f2*, putative legumin; *g*, class III peroxidase 31 precursor; *h*, actin (Os03g50890); *i*, α-1,4-glucan phosphorylase H isozyme; *j1* and *j2*, putative soluble inorganic pyrophosphatase; *k*, putative isocitrate/isopropylmalate dehydrogenase; *l*, putative glyceraldehyde-3-phosphate dehydrogenase; *m*, class III peroxidase 78 precursor; *n*, UDP-glucose pyrophosphorylase. *B1*, putative vacuolar proton-ATPase. *B2*, putative ascorbate peroxidase. *B3*, putative pollen-specific protein C13 precursor. *B4*, β-expansin OsEXPB13. In *C*, *o*, putative exopolygalacturonase precursor; *p*, unknown protein; *q*, putative glyceraldehyde-3-phosphate dehydrogenase. *D1*, expressed protein similar to cell wall protein FLO11p (Os11g11730). *D2*, putative polygalacturonase.

most pollen allergens were glycoproteins, whereas glycan probably contributes to their allergenic activity (46). And Os09g39950 contains the function domain pfam01190 of the Ole e I family, and its homolog Ole e10 from olive pollen tubes has been detected to bind 1,3-β-glucans preferentially (45). In addition, among these detected phosphoproteins (Supple-

TABLE III

Possible PTM/splice variant-derived isoforms in the differentially expressed proteins (these spot numbers correspond to those in Fig. 8)

No.[a]	Protein name	Chr.[b] locus	Theoretical		Known PTMs in Refs.[c]	Spot number[d]	Apparent		Deviation[e]		C[f]	PTM[g]
			MM	pI			MM	pI	MM	pI		
			Da						*Da*	%		
Wall remodeling and metabolism												
1	UDP-glucose pyrophosphorylase	Os02g02560	51,866	5.46	Gly, Pho (70)	-/G601	22,833	4.94	−55.98	−9.52	U	
						P41/G111	58,564	5.46	12.91	0	R	
2	Reversibly glycosylated polypeptide	Os03g40270	41,861	5.82	Gly (71)	P902/G874	31,852	5.99	−23.91	2.92	U	
						P95/G35	48,918	5.61	16.86	−3.61	U	
						-/G119	45,702	5.46	9.18	−6.19	U	Pho
3	TPA: class III peroxidase 78	Os06g20150	35,865	6.25	Gly (42)	P444/G245	38,133	6.23	6.32	−0.32	U	
						P702/G699	31,830	6.27	−11.25	0.32	U	
4	TPA: class III peroxidase 31	Os02g50770	48,315	5.84	Gly (42)	P605/G326	42,429	5.85	−12.18	0.17	U	
						P194/G45	39,021	5.82	−19.24	−0.34	U	
						P15/G273	56,122	5.74	16.16	−1.71	R	Gly
5	Putative exopolygalacturonase	Os02g10300	43,778	7.47	Gly (44)	-/g242	61,131	7.18	39.64	−3.88	U	
						-/g281	57,762	7.31	31.94	−2.14	U	
6	Putative polygalacturonase	Os06g35320	43,088	8.07	Gly (44)	p952/g433	59,671	7.28	38.49	−9.79	R	Gly
						p565/g148	61,272	7.45	42.20	−7.68	R	Gly
						p621/-	61,626	7.05	43.02	−12.64	R	
7	Putative pollen-specific protein C13	Os09g39950	17,830	5.15	Gly (43)	P150/-	20,453	5.11	14.71	−0.78	R	
						P47/G269	22,843	5.14	28.12	−0.19	R	Gly
8	β -Expansin OsEXPB13	Os03g01650	29,228	8.01	Gly (45)	P29/-	35,292	6.73	20.75	−15.98	R	
						P9/G22	36,950	6.51	26.42	−18.73	R	Gly
9	β -Expansin	Os10g40090	28,966	8.66	Gly (45)	p554/g82	38,222	9.32	31.95	7.62	R	Gly
						-/g93	34,715	9.77	19.85	12.82	U	Gly
10	Expressed protein, similar to cell wall protein FLO11p (Os11g11730)	Os11g11710	30,071	4.68	Gly (72)	p616/-	48,954	4.64	62.79	−0.85	R	
						p583/-	49,123	4.32	63.36	−7.69	R	
						p592/-	49,010	4.50	62.98	−3.85	R	
Carbohydrate and energy metabolism												
11	α -1,4-Glucan phosphorylase H isozyme	Os01g63270	91,434	7.36		P240/G47	33,400	5.70	−63.47	−22.55	U	
						-/G89	26,944	5.81	−70.53	−21.06	U	Pho
12	OJ000223_09.15, putative glyceraldehyde-3-phosphate dehydrogenase	Os04g40950	36,921	6.34		P73/G2	45,290	6.79	22.67	7.10	U	Gly
						P75/G16	43,843	6.51	18.75	2.68		
13	Predicted OJ1791_B03.34 gene product, putative glyceraldehyde-3-phosphate dehydrogenase	Os02g38920	36,716	7.68		p771/g45	46,216	8.58	25.87	11.72	U	
						-/g344	22,390	9.63	−39.02	25.39	U	
14	Enolase	Os10g08550	47,942	5.41	Pho (73)	P23/G101	58,297	5.43	21.60	0.37	R	
						P24/G106	57,912	5.29	20.80	−2.22	R	
15	NADP-malic enzyme	Os05g09440	63,294	5.52		P167/G53	67,521	5.60	6.68	1.45	U	
						P567/G527	67,899	5.74	7.28	3.99	U	
16	Putative soluble inorganic pyrophosphatase	Os10g26600	22,256	5.71	Pho (47)	-/G189	27,824	5.55	25.02	−2.80	U	
						-/G42	26,007	5.75	16.85	0.70	U	Pho
						-/G394	26,672	6.28	19.84	9.98	U	
						-/G39	26,146	6.00	17.48	5.08	U	
17	OSJNBa0044K18.22, containing domain pfam00180, putative isocitrate/isopropylmalate dehydrogenase	Os04g40310	36,882	5.77	Pho (74)	P60/G10	44,519	6.22	20.71	7.80	U	Gly
						P58/G14	51,772	6.16	40.37	6.76	U	Gly
Cytoskeleton dynamics												
18	Actin	Os11g06390	41,596	5.31	Pho (75)	-/G54	46,582	5.79	11.99	9.04	U	
						-/G321	39,431	5.51	−5.20	3.77	U	
19	Actin	Os03g50890	41,883	5.29	Pho (75)	-/G121	28,760	5.41	−31.33	2.27	U	
						-/G122	31,322	5.45	−25.22	3.02	U	

TABLE III—*continued*

No.[a]	Protein name	Chr.[b] locus	Theoretical MM	Theoretical pI	Known PTMs in Refs.[c]	Spot number[d]	Apparent MM	Apparent pI	Deviation[e] MM	Deviation[e] pI	C[f]	PTM[g]
			Da						*Da*	%		
						-/G184	31,248	5.49	−25.39	3.78	U	
						P127/G31	52,452	5.30	25.23	0.19	U	
Ion transport												
20	Putative VADC	Os01g51770	33,793	5.39	Pho (76)	P492/G248	46,456	5.26	37.47	−2.41	U	
						P777/G287	47,671	5.24	41.07	−2.78	U	
						P654/G597	33,793	4.92	0	−8.72	U	
21	Putative vacuolar proton-ATPase	Os06g45120	68,711	5.20		P182/-	70,309	5.14	2.33	−1.15	D	Gly; Pho
						P34/G85	67,993	5.11	−1.04	−1.73	D	
Stress response												
22	Putative ascorbate peroxidase	Os03g17690	27,255	5.42		P64/-	30,081	5.29	10.37	−2.40	R	Gly
						P13/-	29,644	5.48	8.77	1.11	R	Gly
						-/G210	28,167	5.52	3.35	1.85	U	
23	Putative legumin	Os05g02520	38,456	5.81	Gly (77)	P254/G77	41,987	5.58	9.18	−3.96	U	
						-/G417	37,187	6.12	−3.30	5.34	U	
						P20/G58	42,570	5.89	10.70	1.38	D	Gly
Unknown protein												
24	Unknown protein	Os09g24120	13,992	7.77		p549/g23	13,977	9.06	−0.11	16.60	R	
						p613/g200	50,441	7.57	260.50	−2.57	R	

[a] Number of the unique proteins with multiple isoforms.
[b] Chromosome.
[c] PTMs reported in references. Pho, phosphorylation; Gly, glycosylation.
[d] Number of protein spots; identical to that in Figs. 4, 5, and 8 and Tables I and II.
[e] Error deviations (%) ((apparent value − theoretical value)/theoretical value × 100%) of apparent MM and pI estimated in 2-DE gels as compared with theoretical values.
[f] Change in abundance of the protein spots on 2DE gel after germination. D, down-regulation; R, probably released to medium; U, up-regulation.
[g] Predicted modifications in this study. Pho, phosphorylation; Gly, glycosylation.

mental Fig. S2B and Table III), soluble inorganic pyrophosphatase has been found to be phosphorylated in the pollen tubes of *Papaver rhoeas* (47). These data suggest that at least glycosylation and/or phosphorylation is involved in the generation of isoforms.

DISCUSSION

Germinated Pollen and Mature Pollen Share a Similar Protein Expression Pattern—Mature pollen from most plants is metabolically quiescent and highly desiccated when released from anthers (48). However, on loading onto the stigma or in a hydrated environment, the pollen can germinate to give rise to a polarly growing pollen tube in a short time. Recent studies of the *Arabidopsis* pollen transcriptome revealed that, compared with early developing microspores (49) and sporophytic tissues (9), mature pollen has a specialized complement of transcripts characterized by a sharp decline in number of diverse transcripts and an increased proportion of pollen-specific mRNAs. The specialized transcriptome appears to be implicated in pollen function specialization. But these observation also show that the pollen transcriptome is underrepresented in transcripts implicated in translation with undetectable ribosomal protein-encoding mRNAs (8).

Recent proteomics analyses from *Arabidopsis* (12, 13) and rice (14) suggest that mature pollen has presynthesized a complement of proteins required for germination and early tube growth. Importantly mature pollen has stored translation-related proteins, including ribosome proteins (12, 14). Together transcriptomic and proteomic data showed that mature pollen has presynthesized these mRNA and proteins to prepare for quick pollen germination and polar pollen tube growth.

In the present study, we screened proteins expressed differentially in MPGs and GPGs by use of 2-DE and MS. 2-DE revealed 1004 ± 22 spots for MPGs and 1030 ± 45 spots for GPGs (pH 4–7 range) with high reproducibility. Of about 2300 protein spots (pH 4–7 range and pH 4–10 part of the pH 3–10 range) in the two samples, 120 spots showed changes in level, whereas only 66 appeared to be sample-specific, suggesting that the changes in development and metabolism were not associated with, at least for the high and medium abundance proteins, appearance or disappearance of proteins. Relatively identical protein patterns were also observed in mature pollen and pollen tubes of Gymnospermae pine (15). Together with the proteomic data of mature pollen of *Arabidopsis* and rice (12–14), our data further indicate that pollen germination and early tube growth depend mainly on these presynthesized proteins in mature pollen.

Preferential Representation of Wall-related Proteins in Pollen Proteome Is an Important Property of Pollen Function Specialization—Although recent transcriptome studies of *Ara-*

bidopsis pollen have not resulted in similar conclusions about the distribution of pollen-expressed transcripts in functional categories (8–10, 49, 50), overrepresented transcripts in the transcriptome are generally considered to be implicated in wall metabolism, cytoskeleton, and signaling (8, 9, 49). Our proteomics analysis revealed that these proteins are involved in not only these three functional categories but also carbohydrate/energy metabolism, protein synthesis and degradation, and stress response (14).

To evaluate the proteomic difference between pollen and sporophytic tissues, we compared proteomic data from mature rice pollen (14) and rice sporophytic tissues, including leaves (811 proteins), roots (1051 proteins), and seeds (702 proteins) identified by Koller *et al.* (51). Comparison of 10 function categories (Supplemental Table S2) revealed that the most important difference between pollen and sporophytic proteomes is the high representation of wall-related proteins in the pollen proteome (11%) (14) but the lowest representation in the sporophytic proteomes (0.25% in leaves, 0.67% in roots, and 0.28% in seeds) (51). Thus, highly active wall dynamics is a prominent property of pollen function specialization.

Just like the mature pollen proteome, the differentially expressed proteins showed a functional skew toward wall and carbohydrate/energy metabolism (Fig. 7*A*). The wall metabolism-related proteins displayed two distinct changed patterns during pollen germination. These up-regulated proteins mainly involve wall synthesis (Table I) and wall hydrolysis/loosening enzymes, such as UDP-glucose pyrophosphorylase, reversibly glycosylated polypeptide, polygalacturonase Os02g10300, class III peroxidases, and β-expansins Os10g40090 and Os03g01640 (Table I). Our previous study revealed that mature rice pollen contains all the enzymes for pectin degradation (14), which suggests that pectin may be important for rice pollen function. Recent evidence has suggested that alternative activity changes in polygalacturonase, as well as pectin methylesterase, mediated by apical proton gradient are required for polar growth of the tube (7). Polygalacturonase in *Aspergillus niger* has a role in stimulating pollen germination and tube growth (52). Therefore, these proteins, up-regulated on germination, might be mainly responsible for fast pollen tube growth. In contrast, down-regulated wall-related proteins such as β-1,4-xylanase, polygalacturonase Os06g35320, β-expansin OsEXPB13, and one isoform of β-expansin Os10g40090 were mainly involved in wall degradation and loosening (Table II). The down-regulation of most of these proteins should result from their release from the germinating pollen into the medium (Table II), which is possibly similar to their situation *in vivo*. In the monocots maize and rice, β-1,4-xylanase is the major protein component in the pollen coat (14, 53) and possibly facilitates pollen tube invasion by hydrolyzing the xylan on the stigma surface (53). Besides being responsible for degradation of demethylesterified pectin (7), polygalacturonase-mediated release of oligogalacturonides can act as a signal for different development processes (54). The *in vivo* release of these proteins might be necessary for pollen tube invasion growth in pistils.

Selective Degradation of Proteins May Be Vital for Fast Pollen Tube Growth—Mature rice pollen has presynthesized a complement of translation-related proteins with multiple molecular chaperones (14). After germination, TCTP, an effective inhibitor of translation elongation (38), was down-regulated (Table II), whereas eIF4A and eIF4G, involved in protein translation regulation at the level of ribosome recruitment, were greatly up-regulated (Table I). This finding suggests that active protein synthesis is initiated in growing tubes.

Besides the regulation of protein synthesis, the temporal regulation of protein function is vital to cellular and developmental processes as much evidence has suggested. 26 S proteasome-based selective protein degradation is an important mechanism for temporal regulation and involves numerous cellular and development processes, including pollen-stigma interaction (4) and polarized cell morphogenesis (55). The disruption of pollen germination by inhibitors of 26 S proteasome activity (56) and presynthesis of multiple subunit components of the complex in mature rice pollen (14) suggest that the pathway is implicated in regulation of pollen function. Furthermore our differential proteomics analysis identified six of 14 subunits of the 20 S proteolytic particle of the proteasome, all of which were up-regulated in growing tubes (Table I) as were some peptidases. Therefore, 26 S proteasome-mediated protein degradation might be an important regulator of pollen function.

The Presence of Multiple Isoforms Underlines the Importance of Their Function in Pollen Germination and Tube Growth—Besides closely related sequences from different genes and splice variants from a gene, more than 200 PTMs contribute to the generation of isoforms (57). Increasing data from the studies of animal cells show that isoforms have different subcellular localization (58, 59) and/or functions (57). Therefore, isoforms are generally considered to diversify the function of a protein. Recent proteomics studies have revealed that isoforms are common in analyzed seeds (60), anthers (61), and mature pollen (12–14). In our identified Unipros from mature rice pollen, 23% have isoforms (14) with wide distribution in different functional categories (Fig. 9*B*). A similar proportion was found in the identified proteins from mature *Arabidopsis* pollen (13), implying the functional importance of isoforms.

Consistently about 25% of the differentially expressed Unipros we identified have isoforms with a distribution in functional categories (Fig. 9*A*) similar to those identified in mature rice pollen (Fig. 9*B*). Six (5%) of these protein species, actin, α-tubulin, β-tubulin, polygalacturonase, β-expansin, and VDAC, have sequence-related isoforms (Fig. 8 and Tables I, II, and III). In animal sperm cells, isoforms of tubulin participate in different microtubulin structures, thus possibly having differ-

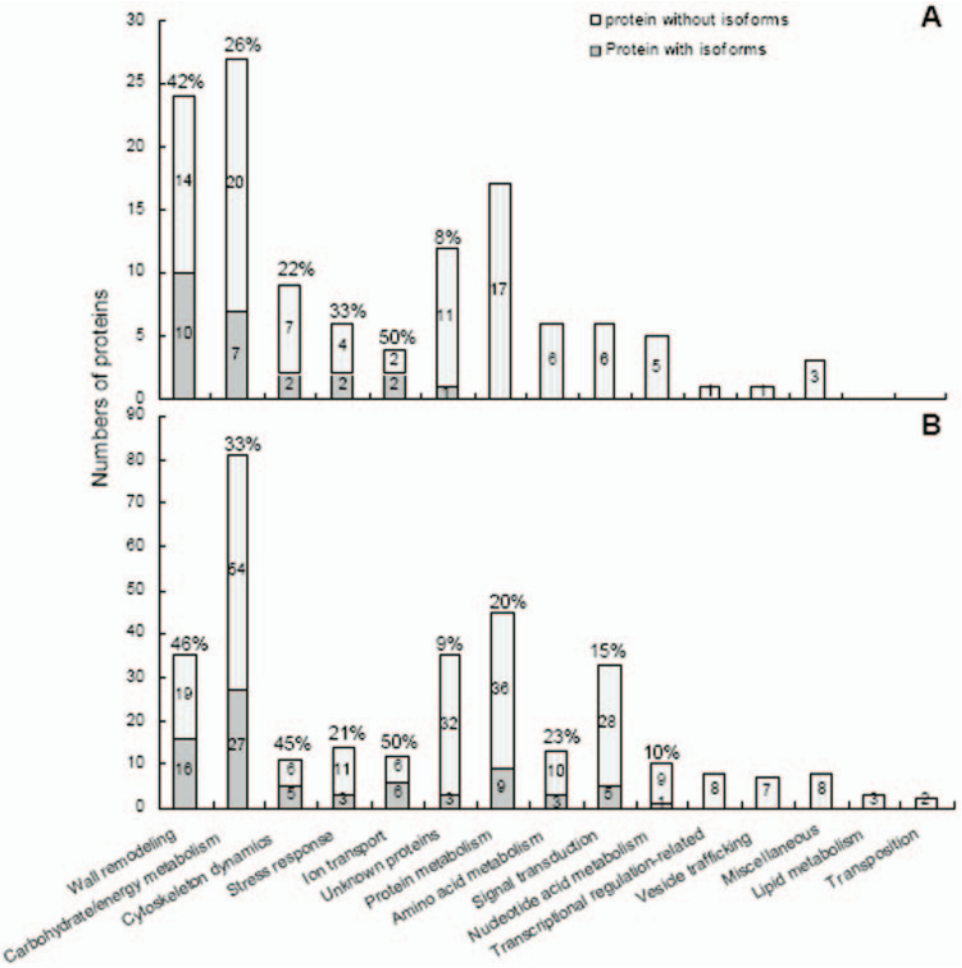


FIG. 9. **Distribution of our differentially expressed protein species with isoforms by function (*A*) and those identified from mature rice pollen grains (14) (*B*).** Each *column* shows number of proteins with (*black*) and without (*dash dotted*) isoforms. The percentages of proteins with isoforms in each category is at the *top*.

ent functions (62). *Arabidopsis* pollen-expressed pectin methylesterase isoforms appear to have different functions for pollen germination and pollen tube growth (7). Our data show that the sequence-related isoforms had distinct expression patterns in rice pollen (Tables I, II, and III) during the developmental switch from mature pollen to germination and tube growth. For example, polygalacturonase Os02g10300 newly appeared, whereas Os06g35320 was probably released in the growing tubes (Table III). Furthermore the 24 protein species giving rise to isoforms possibly by PTMs/splice variants appeared as 59 identities (Fig. 8 and Table III); 37 of these were up-regulated, and 22 were down-regulated or possibly released during pollen germination and tube growth. Interestingly for proteins such as β-expansin and class III peroxidase 31, some isoforms were up-regulated, and others were down-regulated (Table III), suggesting their different function.

In addition, our results preliminarily demonstrate that at least glycosylation and/or phosphorylation is involved in the generation of some isoforms. The importance of these predicted modified proteins in pollen tube growth remains largely unknown, but some evidence indicates that glycosylation is required for subcellular localization and transportation of proteins (63). This process might be important for wall loosening/hydrolysis-related proteins such as polygalacturonase and β-expansin, which have roles in facilitating pollen tube invasion growth in pistils by localizing around the tube wall and/or being released into tract tissues of the pistil. Soluble inorganic pyrophosphatase is involved in the generation of ATP and synthesis of biopolymers; the latter are material for cell membranes and walls. In the self-incompatibility response of *Papaver rhoeas*, Ca^{2+} gradient-mediated hyperphosphorylation of this protein in the apical pollen tube was proposed to be associated with the inactivity or down-regulated activity of the enzyme, consequently resulting in inhibition of pollen tube growth (47). Our results showed that this protein has multiple up-regulated isoforms and that one of them is phosphorylated in the germinated rice pollen, suggesting a strict functional regulation of this protein. All this evidence demonstrates the

importance of isoforms in pollen germination and tube growth, although direct information about function is still lacking.

In summary, we have established an *in vitro* germination method for mature rice pollen and screened 186 protein spots differentially expressed in mature and germinated pollen by 2-DE-based differential proteomics approaches. Most of the proteins showed differences in level between the two proteomes, and only 66 were specific to development stage. Furthermore 160 differentially expressed protein spots were identified on MS to match with 120 diverse protein species. These differentially expressed proteins showed an obvious functional skew, like those in the mature pollen proteome (14). Wall metabolism-related proteins are predominant in the pollen proteome as compared with rice sporophytic proteomes (51) (Supplemental Table S2). The study has also revealed multiple isoforms in the differentially expressed proteins and different expression patterns between isoforms of a protein, a finding not achieved with transcriptomics approaches. These results have provided novel insights into pollen function specialization important for understanding the molecular regulation of polar tube growth.

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