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ORIGINAL ARTICLE

Proteome profile and functional classification of proteins in *Arabidopsis thaliana* (Landsberg erecta) mature pollen

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Abstract Proteome analysis of mature *Arabidopsis thaliana* (Landsberg erecta ecotype) pollen was conducted using two-dimensional gel electrophoresis and mass spectrometry. A total of 960 spots were resolved on pH 4–7 IPG strips and 110 distinct proteins were identified from 150 spots analyzed. The identified proteins were categorized based on their functional role in the pollen, which included proteins involved in energy regulation, defense-related mechanisms, calcium-binding and signaling, cytoskeletal formation, pollen allergens, glycine-rich proteins (GRPs), and late embryogenesis abundant (LEA) proteins. These proteins potentially play important roles in pollen function at maturity and during subsequent germination and tube growth. Some of the proteins identified were related to known pollen-specific transcripts, while some were similar to proteins found in the seed. In this study, 66 new proteins were identified which were not reported in two other recent studies on *Arabidopsis* pollen, 17 proteins were common in all three studies, and 35 or 26 proteins reported here had an overlap with one or the other two studies. These differences may be attributed to the methods of protein extraction, spot selection for analysis, and the ecotype used. Together, the three studies provide a broad spectrum of the *Arabidopsis* pollen proteome.

Keywords *Arabidopsis thaliana* · Calcium-binding proteins · LEA proteins · Pollen · Proteomics

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Introduction

The pollen grain plays an essential role in sexual reproduction of higher plants, with its primary function of delivering sperm cells to the female gametophyte via the formation of a pollen tube. In angiosperms, the pollen grain is a relatively simple two- or three-celled structure produced in the anther of a stamen. The development of pollen is highly controlled and follows a precise pattern of sporogenesis and gametogenesis, with some differences between dicots and monocots at the time of meiosis of pollen mother cells (Bedinger 1992; Palmer et al. 1992).

Genetic control of anther and pollen development has been investigated in a number of species, including *Arabidopsis* (Mascarenhas 1990). A large number of genes controlling stamen and pollen development have been identified using a variety of approaches, including mutant isolation, mRNA expression, and in situ hybridization (Sanders et al. 1999; McCormick 2004; Scott et al. 2004). Both nuclear and cytoplasmic (mostly mitochondrial) genes regulate pollen development (Hanson and Betolila 2004), and approximately 20,000 genes are expressed during this process of which about 10% are specifically expressed in the pollen (Hamilton and Mascarenhas 1997; Honys and Twell 2003). Detailed transcriptomic analyses of *Arabidopsis* pollen

have revealed that a majority of genes expressed in the pollen have an overlap with sporophytic tissues, but several genes are either enriched or specifically expressed in the pollen (Becker et al. 2003; Honys and Twell 2003; Pina et al. 2005). As well, the most prevalent pollen-expressed genes are involved in signaling, vesicle transport, and the cytoskeleton.

We extend these studies to analyze the *Arabidopsis* pollen proteome and to classify proteins based on their functional roles. An understanding of the pollen proteome would provide insight into the relationship between the transcriptome in mature pollen and certain specialized proteins that are readily available for pollen germination and tube growth. In addition, since the pollen grain, like the seed, is a dispersal agent of higher plants that is capable of becoming highly desiccated and dormant, there are possible similarities between the pollen (Holmes-Davis et al. 2005; Noir et al. 2005; Dai et al. 2006) and seed (Gallardo et al. 2001; Sheoran et al. 2005; Vensel et al. 2005) that are reflected in the proteome.

Two articles have recently appeared on the *Arabidopsis* pollen proteome in which different methods of protein extraction were applied to the Columbia ecotype (Holmes-Davis et al. 2005; Noir et al. 2005). Although some of the identified proteins are common between these studies, many proteins are different in at least two of the three studies. Taken together, the results of these three independent investigations provide a firm basis of technological and biological sampling and provide a broad analysis of the *Arabidopsis* pollen proteome. This study also reports on a number of proteins that were not reported earlier in *Arabidopsis* pollen.

Materials and methods

Plant material and growth conditions

Seeds of the wild type *Arabidopsis thaliana* ecotype Landsberg erecta were originally provided by Dr. B. Mulligan of the University, Nottingham, UK (Fei et al. 2004). Seeds were placed in 15 cm plastic pots containing Tera-lite Redi-earth mix and were stratified by exposing the pots to 15°C in the dark for 3 days. Plants were subsequently grown at 22/20°C (day/night) and 16/8 h light/dark in a growth chamber. Light was provided by fluorescent tubes (Osram Sylvania Versailles, KY, USA) at 120–150 μM m^{-2} s^{-1}.

Pollen collection

Flowers at anthesis were picked daily, and placed in open micro-centrifuge tubes overnight at 4°C to promote anther dehiscence. Flowers were then rinsed at least four times with acetone in the tubes to obtain a pollen suspension, which was transferred to fresh tubes using a pipette. The pollen suspension was centrifuged, the supernatant removed, and the pellet transferred onto a glass slide. Pollen samples were checked under a dissecting microscope, and pooled following the removal of any debris. The purity of isolated pollen was determined by light microscopy, and the pollen was stored at –80°C until further use. Two separate batches of clean and pooled samples were used for protein extraction. Pollen viability of each of the two batches of freshly colleted pollen was tested by in vitro pollen germination test (Shivanna and Sawhney 1995) and it ranged from 70 to 75%.

Protein extraction

The pollen (approximately 20 mg) was ground with a glass rod in a micro-centrifuge tube with cold 10% TCA and 1% DTT in acetone and kept at –20°C for at least 2 h. The samples were centrifuged at 25,000 *g* for 20 min at 4°C, and the resulting pellet was washed by suspending in acetone containing 1% DTT, incubated at –20°C for 2 h, and centrifuged. The pellet was suspended again in acetone, sonicated (3 × 15 s), and centrifuged at 25,000 *g*. The pellet was vacuum dried and total soluble proteins were extracted by dissolving in iso-electric focusing (IEF) compatible buffer comprising 8 M urea, 20 mM DTT, 4% CHAPS, and 2% ampholyte (pH 3–10). The solution was vortexed extensively for 1 h at room temperature, centrifuged at 20°C for 20 min at 25,000 *g*, and the supernatant was collected. The resulting pellet was resolubilized and vortexed for 1 h, centrifuged at 25,000 *g*, and the supernatant combined with that collected earlier. The resulting protein samples were centrifuged again for 20 min at 25,000 *g*. Total soluble protein in the supernatant was estimated with Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) and used immediately for further analysis or stored at –80°C for later use. The protein content in the samples ranged between 95 and 120 μg/mg fresh wt.

Two-dimensional gel electrophoresis

Two-dimensional electrophoresis (2-DE) was conducted according to Sheoran et al. (2005). IEF was performed using the Multiphor II horizontal electrophoresis system and 13 cm Immobiline Dry Strips of 4–7 or 3–10 linear pH gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). The strips were rehydrated overnight in a solution containing 8 M urea,

2% CHAPS, 20 mM DTT, 0.002% bromophenol blue, 2% IPG buffer (pH 3–10), and 600 μg of the protein sample. IEF was carried out by applying a voltage of 250 V for 1 h, increasing to 3,500 V over 2 h, and holding at 3,500 V until total of 75 kVh was obtained.

Following IEF, the strips were equilibrated for 15 min in an equilibration buffer containing 0.05 M Tris–HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.125% (w/v) DTT, followed by another 15 min equilibration in the same buffer containing 125 mM iodoacetamide without DTT. The equilibrated strips were applied to vertical SDS-polyacrylamide gels (12.5% resolving 5% stacking) and sealed with 0.5% agarose in SDS buffer containing bromophenol blue. Electrophoresis was performed for 30 min at 25 mA, and then for 3 h at 40 mA in SDS electrophoresis buffer containing 25 mM Tris base, 192 mM glycine, and 0.1% SDS, pH 8.3.

Gel staining

Gels were fixed in 50% ethanol with 10% orthophosphoric acid over-night, washed with water (3×20 min), and stained in a Colloidal Coomassie Blue G-250 (CCB) solution (0.12% CB, 10% ammonium sulfate, 10% orthophosphoric acid, 20% methanol; Candiano et al. 2004) for 2 days. After washing with water, gels were scanned, annotated, and analyzed for spot number and spot volume using Phoretix 2D Image analysis software (UBI, Canada). Three replicate gels were run for each of two different pollen samples, and protein spots observed consistently in replicate gels were selected for further analysis.

Mass spectrometry

Of the 960 spots observed consistently in stained gels, 150 spots were selected across the gel representing different molecular weights and PIs for identification by mass spectrometry. These spots were excised using a Protean 2-D spot cutter (Bio-Rad), and placed in a 96-well microtitre plate (Sigma, Milwaukee, WI, USA). Excised proteins were automatically de-stained, dehydrated, reduced with DTT, alkylated with iodoacetamide, and digested with trypsin using a MassPREP protein digest station according to the recommended procedures (Micromass, Manchester, UK). Mass spectra of the resulting tryptic digests were acquired by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) on a Voyager-DE STR (Applied Biosystems, Framingham, MA, USA) as previously described (Sheoran et al. 2005). Five micro liter of each digest were dried to approximately 1 μl on a MALDI target plate. One micro liter of α-cyano-4 hydroxy-cinnamic acid matrix solution (5 mg/ml in 0.1% TFA/75% acetonitrile) was then added to each sample, and the mixture allowed to air dry. The instrument was calibrated using trypsin autolysis products (*m/z* 842.51 and 2,211.10) as internal standards, or a mixture of des-Arg bradykinin (*m/z* 904.4681) and ACTH clip 18–39 (*m/z* 2,465.1989) for close external calibration. Peak lists generated from the tryptic digest spectra were submitted for peptide mass fingerprinting (PMF) using MASCOT (http://www.matrixscience.com/) search engine and NCBI non-redundant protein database (November, 2005). The following parameters were used for database searching: carbamidomethylation of cysteine (fixed modification); oxidation of methionine (variable modification); one missed cleavage (trypsin); and a mass deviation of less than 50 ppm. For proteins that could not be identified by PMF, the remainder of the digest was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a capLC system and Q-Tof Ultima Global mass spectrometer (Waters-Micromass, Milford, MA, USA) as previously described (Sheoran et al. 2005). LC-MS/MS data were processed using ProteinLynx v2.15 software (Micromass) and searched against NCBI non-redundant protein database using the MASCOT search engine. Proteins identified with a MOWSE score greater than 70 (95% confidence interval) are reported. Functional categorization and sub-cellular localization of identified proteins was performed using The *Arabidopsis* Information Resource (TAIR) database (http://www.arabidopsis.org).

Results and discussion

On average, 960 spots were observed on a 2-D gel stained with CCB using pH 4–7 IPG strips (Fig. 1). Of the 150 spots analyzed using both MALDI-TOF MS and LC-MS/MS, 132 spots representing 110 distinct proteins were identified by searching various publicly available protein databases. Table 1 lists each identified protein by its gene index (gi) number and the corresponding AGI gene locus, as obtained from TAIR. In most cases, the calculated molecular mass and pI of identified proteins were close to the theoretical values (Table 1). Multiple spots corresponding to the same protein were also identified, as has been reported in other proteomic studies. Deviations in molecular mass and pI, and the presence of multiple spots corresponding to the same protein, could be due to a number of factors, including post-translational

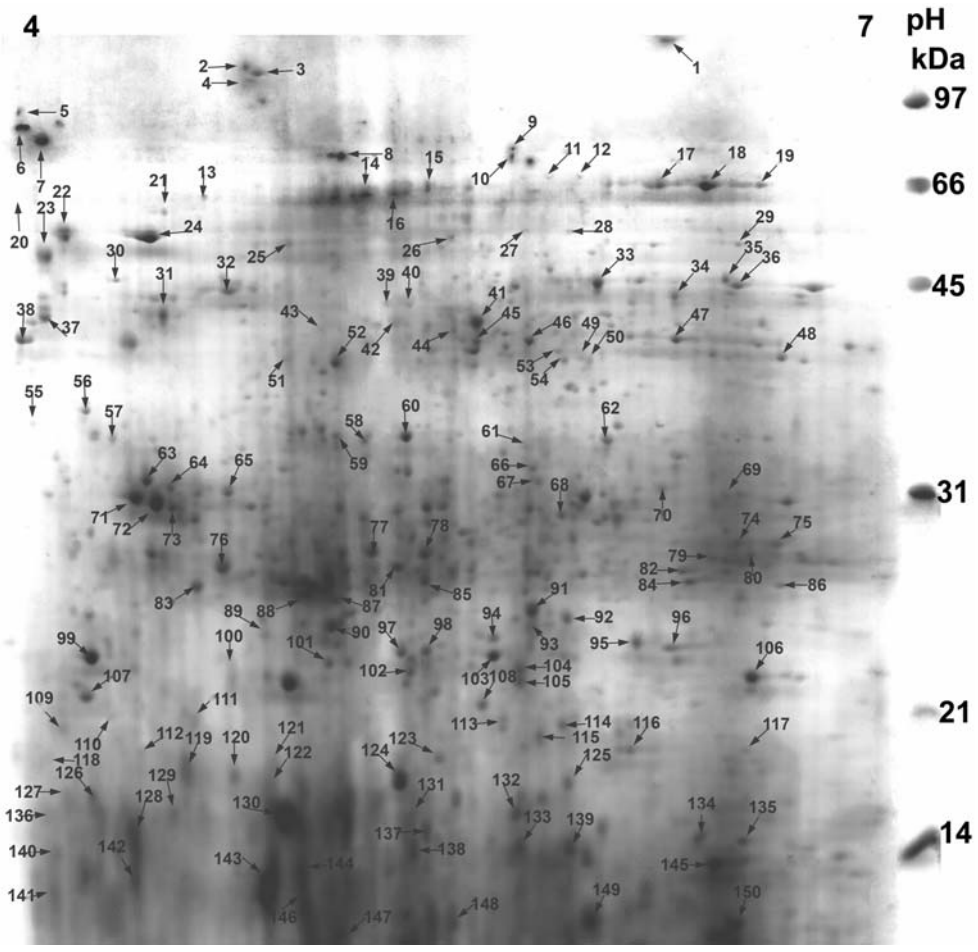

Fig. 1 Colloidal Coomassie stained 2-DE gel (pH 4–7) of mature *Arabidopsis* pollen. *Spot numbers* indicated on the gel were subjected to MALDI and/or LC- MS/MS analysis. Standard molecular weight markers are shown on the *right*

modifications (e.g., subsequent addition of phosphate, methyl, or other groups), protein degradation, partial synthesis of proteins during pollen maturation, or protein translation from alternatively spliced mRNAs.

Pollen is an abundant source of protein, with protein content ranging from 2.5 to 61% (Roulston et al. 2000). Pollen proteins identified in this study are likely to be predominent in the vegetative cell, since sperm cells contain few organelles and a small cellular volume, e.g., less than 0.05% of the pollen volume in *Plumbago* (Russell and Strout 2005), and proportionately fewer transcript copies (Xu et al. 1999; Engel et al. 2003). Using the Gene Ontology annotation for *Arabidopsis* (Berardini et al. 2004), the relative distribution of identified proteins (Table 1) associated with various cell components is presented in Fig. 2. Mitochondrial proteins accounted for the largest single group (approximately 29%), followed by proteins in the cytosol (10%). Other cellular sources include ER (7%), nucleus (6%), and cell wall (5%). About 19% of protein products could not be assigned to a specific cellular component. Noir et al. (2005) reported that the largest protein fraction in the pollen of *Arabidopsis* Columbia ecotype to be cytoplasmic (41%), with 40% targeted to the endomembrane system. We observed more pollen proteins associated with the cell wall than did Noir et al. (2005), which may, in part, be due to different methods of protein extraction.

Functional classification of identified proteins

The identified proteins (Table 1) were classified into functional groups (Fig. 3) based on putative protein functions (Berardini et al. 2004). The two major groups of identified proteins in *Arabidopsis* pollen were those involved in energy regulation (18%) and those involved in defense- and stress- response (20%). Other well-represented functional categories were protein and other metabolism (9% each), cytoskeleton (8%), transport (8%), and transcriptional regulation (4%). We separated Ca^{2+}-binding and signaling (5%), pollen allergen (6%), and LEA proteins (5%) into separate groups because of their physiological significance in the pollen. The other two recent studies (Holmes-Davis et al. 2005; Noir et al. 2005) also suggest that the majority of proteins

Table 1 *Arabidopsis* (Landsberg erecta) mature pollen proteins identified by MALDI-TOF MS or LC-MS/MS

Spot number[a]	Gene index number	AGI gene locus	Protein identity	kDa/pI[b]	Sequence coverage (%)	Mascot score
1	7268300	At4g15530	Pyruvate phosphate dikinase family protein[d]	104.9/5.8	23	108
2	15222149	At1g49490	Structural constituent of cell wall	91.2/4.9	28	147
3	15222149	At1g49490	Structural constituent of cell wall	91.2/4.9	23	69
4	15222149	At1g49490	Structural constituent of cell wall	91.2/4.9	15	89
5	30681829	At2g22795	Unknown protein	82.0/4.3	45	354
6	30681829	At2g22795	Unknown protein	82.0/4.3	30	210
7	6681343	At3g09840	Cell division cycle protein 48[c,d]	89.3/5.0	65	413
8	1303695	At5g28540	Luminal binding protein (BiP)[c]	73.7/5.1	40	1,029
9	18414718	At4g16660	ATP binding	96.7/5.9	19	295
10	15293149	At1g11660	HSP protein	96.9/5.8	15	250
11	30697452	At5g61030	RNA binding/protein transporter	49.4/5.7	33	360
12	21553535	At5g60980	Ras-GTPase-activating protein (NTF2)	49.4/5.7	41	405
13	15223975	At1g77510	ATPDIL1-2/disulfide isomerase	56.6/4.9	22	196
14	11994364	At3g13930	Dihydrolipoamide acetyltransferase	60.1/7.5	13	76
16	30725440	At1g78900	Vacuolar ATP synthase subunit A[c,d]	68.8/5.0	66	291
17	10177326	At5g65690	Phosphoenol- pyruvate carboxykinase[d]	68.7/6.0	44	186
19	5107826	At5g25880	Putative malate oxidoreductase[c,d]	64.6/6.6	26	164
20	30696733	At5g56360	Calmodulin binding	74.1/4.7	10	98
21	15219086	At1g21750	ATPDIL1-1/disulfide isomerase[c,d]	55.8/4.8	50	965
22	30725696	At1g56340	Calreticulin[d]	48.7/4.5	47	642
23	21555174	At1g09210	Calreticulin[d]	48.4/5.9	54	611
24	23308191	At5g39570	Expressed protein (glycine rich protein)	43.6/4.7	42	178
25*	52354239	At1g79120	Hypothetical protein (DUF 860 domain)	47.5/6.0	27	67
26	13548325	At5g08670	ATP synthase beta-chain[c]	59.8/6.2	48	376
27	20148521	At1g23190	Phosphoglucomutase[d]	63.1/5.9	67	261
28	20148521	At1g23190	Phosphoglucomutase[d]	63.1/5.9	49	197
29*	15221044	At1g48030	Dihydrolipoyl dehydrogenase (F21D18028)	54.0/7.0	37	80
30	23308191	At5g39570	Unknown	43.6/4.7	34	273
31	1303695	At5g42020	Luminal binding protein (HSC-70)[c]	73.7/5.1	24	593
32	16323374	At5g44340	Tubulin beta-4 chain[c,d]	49.8/4.6	39	106
33	20453235	At1g65930	Isocitrate dehydrogenase[c,d]	45.7/6.1	44	179
35*	9294498	At3g17940	Aldolase 1-epimerase-like protein[d]	37.3/5.9	29	79
36	7529717	At3g52930	Fructose bisphosphate aldolase[c,d]	38.9/6.1	27	214
37	21555174	At1g09210	Calreticulin[d]	48.4/4.4	22	143
38	21555174	At1g09210	Calreticulin[d]	48.4/4.4	35	261
39	4309733	At2g18340	Similar to LEA protein	49.8/5.5	29	176
41	16226235	At2g36530	F1011.16/enolase[c,d]	35.0/5.2	10	115
42a	17939849	At5g08670	Mitochondrial ATP synthase beta subunit[c]	63.6/6.5	20	355
42b	28393806	At5g59370	Actin 4[d]	41.9/5.3	16	188
44	4586118	At4g12130	Putative protein (glycine cleavage T family/ aminomethyl transferase family protein)	40.5/8.7	32	176
45	877848	At1g55700	F20N2.12 (DC-domain containing protein)	77.8/6.5	13	177
46	24417274	At2g47470	Unknown protein (disulfide isomerase)[c,d]	39.8/5.8	34	320
47	4454472	At2g20760	Expressed protein (unknown protein)	37.2/5.9	25	102
48	12642848	At1g53240	Mitochondrial NAD-dependent MDH	36.0/8.5	31	111
49	8778388	At1g13890	F16A14.10 (Synaptosomal-associated protein)	29.1/5.8	47	392
50	8778388	At5g61210	F16A14.10 (Synaptosomal-associated protein)	29.1/5.8	47	442
51	21554576	At3g53750	Actin 3[d]	41.7/5.2	34	98
52	46931236	At4g36600	Similar to LEA protein	37.6/5.3	29	652
53*	42571269	At2g47470	Thioredoxin family protein[c,d]	29.5/5.5	25	88
55*	20465723	At1g22450	Putative cytochrome-C oxidase subunit	21.4/4.3	23	76
56	1695717	At1g09080	Luminal binding protein	73.0/5.0	24	268
57	24496493	At5g02490	BiP chaperone BIP-L	75.3/4.9	12	362
58	24417274	At2g47470	Unknown protein (disulfide isomerase)[c,d]	39.9/5.8	39	475
59	24417274	At2g47470	Unknown protein (disulfide isomerase)[c,d]	39.9/5.8	18	122
60*	15226610	At2g47470	ATPDIL2-1 electron transporter[c,d]	40.0/5.8	35	144
61	8777485	At3g15020	NAD-dependent MDH	36.0/8.3	42	782
62	7769871	At1g53240	F12M16.14 (NAD-dependent MDH)	37.2/8.5	34	502
63*	2083278	At1g35720	Annexin 1[d]	37.8/5.2	66	125
64*	21280929	At5g16510	Reversiably glycolated polypeptide[d]	38.6/4.9	39	75

Table 1 continued

Spot number[a]	Gene index number	AGI gene locus	Protein identity	kDa/pI[b]	Sequence coverage (%)	Mascot score
65	7671424	At5g09650	Inorganic pyrophosphatase	33.6/5.7	10	98
66	8777485	At3g15020	NAD-dependent MDH	36.0/8.3	63	692
68	16173	At1g07890	L-ascorbate peroxidase (APX1)[c,d]	27.8/5.7	13	70
69	15225402	At2g45080	T14P1.11 (cyclin protein)	26.1/4.4	19	67
70	21554322	At1g07890	L-ascorbate peroxidase[c,d]	27.8/5.7	50	500
71*	62320230	At5g28540	Luminal binding protein	15.0/4.4	39	75
72*	62320230	At5g28540	Luminal binding protein	15.0/4.4	43	115
73*	22006985	At3g28340	Glycosyl transferase	41.0/4.4	30	115
74*	30690772	At2g47730	Glutathione S-transferase 6 (GST6)[c,d]	29.3/8.5	38	80
75a	2651722	At4g13560	Unknown protein (similar to LEA 3)[d]	11.6/6.7	45	295
75b	25090885	At3g06050	Thioredoxin reductase[c,d]	21.5/8.9	40	274
76	11270444	At3g55440	Triosephosphate isomerase[d]	27.1/5.1	72	217
77	21554322	At1g07890	L-ascorbate peroxidase[c,d]	27.8/5.7	41	348
78	19310625	At2g21870	Putative ATP synthase[c,d]	27.6/6.3	30	475
80*	21553457	At3g10920	Putative (Mn) superoxide dismutase[c]	25.0/8.5	32	67
81*	19310625	At2g21870	Expressed protein (ATP synthase)[c,d]	27.6/6.3	35	336
82	21593056	At1g75270	Dehydroascorbate reductase	23.5/6.0	41	354
83	26452310	At5g27980	Embryonic abundant protein (LEA protein)	19.6/4.9	40	236
84	18403457	At3g22600	Lipid binding protein	21.1/6.1	12	88
86	60543359	At2g21060	Glycine-rich protein 2 (GRP-2)	19.4/6.3	44	179
87	21554322	At1g07890	L-Ascorbate peroxidase 1[c,d]	27.5/5.7	60	284
91	21593056	At1g75270	Dehydroascorbate reductase	23.4/6.0	23	770
92	196965	At2g47650	dTDP-glucose 4,6-dehydratase[c]	50.0/8.9	9	97
95	18387457	At3g06050	Antioxidant/peroxidase[c,d]	21.5/5.9	23	271
96a	20466103	At3g06050	Unknown (Alkyl hydroperoxide reductase)[c,d]	21.3/9.0	34	147
96b	6714406	At3g05930	Germin-like protein[c,d]	23.1/8.8	15	135
97	20197320	At2g46860	Inorganic pyrophasphatase[d]	24.9/5.5	31	185
99	26450755	At4g20780	Calcium-binding protein	21.2/4.6	38	301
101	16173	At1g07890	L-ascrobate peroxidase (APX1)[c,d]	27.8/5.7	24	170
102	30690243	At5g26667	Uridylate kinase[c]	23.3/6.4	28	178
103a	19310625	At2g21870	Putative ATP synthase[c,d]	27.6/6.3	30	457
103b	12083342	At1g16470	Multicatalytic endopeptidase	25.7/5.5	48	275
104	30690246	At5g26667	Uridylate kinase[c]	22.6/5.8	57	370
105a	17104709	At1g73230	RNA polymerase B transcription factor 3	18.0/5.9	58	262
105b	2497486	At5g26667	Uridylate kinase[c]	22.6/5.8	41	247
106	7267860	At4g11600	Phospholipid hydroperoxide glutathione peroxidase [c,d]	18.7/6.6	27	120
107	1303695	At5g42020	Luminal binding protein (HSC-70)[c]	73.7/5.1	10	151
108	14532850	At4g38680	Glycine-rich protein 2 (GRP-2)	19.5/5.6	10	82
109*	42570833	At2g20630	Protein phosphatase 2C putative	32.1/5.9	27	71
110	25403253	At1g24620	F21J9.28 (similar to polcalcin)	20.4/4.6	71	398
111	48310598	At4g02550	Pectin methylesterase inhibitor (PMEI)	20.0/4.9	21	76
112	25403253	At1g24620	F21J9.28 (similar to polcalcin)	20.4/4.6	27	110
113	20197312	At2g47730	Glutathione S-transferase [c,d]	19.8/5.3	28	195
114	11762128	At4g17530	Ras-related GTP-binding protein (RAB1C)[c]	24.8/5.8	23	72
115	26452507	At5g16450	S-adenosylmethionine 2-dimethylmenaquinone methyltransferase	18.1/5.4	31	129
116	21593482	At4g24640	Pectin methylesterase inhibitor (Bnm1like protein)[c,d]	20.1/5.5	66	351
117	7267860	At4g11600	Phospholipid hydroperoxide glutathione peroxidase[c,d]	18.7/6.6	27	143
118	15221781	At1g24620	Calcium-binding pollen allergen (polcalcin)	20.4/4.6	27	110
119	16203	At5g20230	Blue-copper binding protein[c]	20.2/4.7	9	89
120	17939851	At3g52300	Putative protein (ATP synthase D chain)[d]	19.6/5.1	11	83
121	21555349	At3g52300	F0-ATP Synthase D chain[d]	19.6/5.1	62	515
122a	21553949	At2g30410	Tubulin folding cofactor A	12.9/5.0	42	310
122b	21555349	At3g52300	F0-ATP Synthase D chain[d]	19.6/5.1	59	296
123	4914438	At2g21690	Glycine-rich RNA binding protein 8	16.5/5.6	39	270
124*	4914438	At2g21690	Glycine-rich RNA binding protein 8	16.5/5.6	51	72
125*	21537051	At1g26630	Initiation factor 5A-2	17.4/5.6	33	76
126	21553555	At2g22170	Lipoxygenase (dehydration stress—induced)	20.3/5.1	9	77
127	228408	At1g66400	Calmodulin 1	15.5/4.2	65	262
129	2021593095	At2g27710	60S ribosomal protein P2	11.4/4.8	23	198

Table 1 continued

Spot number[a]	Gene index number	AGI gene locus	Protein identity	kDa/pI[b]	Sequence coverage (%)	Mascot score
130	21593328	At3g53750	Actin 11[d]	42.0/5.3	10	127
131	21593191	At1g47980	Dessication-related protein, putative	34.4/8.7	12	163
132	21553354	At2g21660	Glycine-rich RNA binding protein 7	16.9/5.9	78	310
133*	21618254	None	Unknown protein	11.8/5.4	73	74
136	16225	At2g27030	Calmodulin	16.9/4.2	30	148
137	12323093	At3g62730	Dessication-related protein 70055–71849	33.0/8.3	12	153
138	9802567	At1g08840	F2203.32 (superoxide dismutase)	14.8/5.4	19	120
139	5103841	At1g15415	F9L1.37 (similar to LEA)[d]	10.4/5.9	44	74
140	16225	At2g27030	Calmodulin	16.9/4.2	30	196
141	16223	At1g66400	Calmodulin	16.9/4.2	30	188
142	21592567	At1g80230	Cytochrome C oxidase subunit	18.9/5.4	32	193
143	21536544	At2g19770	Profilin 4	14.1/5.0	17	153
144	21553773	At2g19760	Profilin 3/profilin 4	14.6/5.0	57	258
145	28827234	At4g13560	LEA-domain containing protein[d]	11.6/7.7	87	150
146	21537389	At4g29340	Profilin 3[c,d]	14.6/5.0	46	191
147	4115366	At1g46696	Unknown protein (DUF 601 conserved domain)	47.1/5.9	36	265
148	28827234	At4g13560	Unknown protein (containing LEA domain)[d]	11.6/7.7	45	149
149a	55978673	At1g04670	Hypothetical protein[d]	13.5/9.3	26	89
149b	71633	At3g53750	Actin fragment[d]	42.0/5.3	13	73

[a] Protein spot numbers with asterisk (*) were identified by MALDI-TOF MS and the rest by LC-MS/MS analysis. The annotation of spot number with 'a' and 'b' indicates that two different proteins were identified within that spot. No significant matches were found for the spot numbers not listed in the table

[b] Molecular mass and pI of identified proteins

[c] Proteins reported by Holmes-Davis et al. (2005)

[d] Proteins reported by Noir et al. (2005)

expressed in mature *Arabidopsis* pollen are involved in energy and general metabolism, and in defense against biotic or abiotic stresses. Studies of the Columbia ecotype revealed three spots as Ca^{2+}-binding proteins and four spots as LEA proteins (Noir et al. 2005), and none in data provided by Holmes-Davis et al. (2005). Our study, however, revealed 11 spots representing Ca^{2+}-binding proteins and six spots as LEA proteins. Interestingly, other studies on the pollen proteome of *Arabidopsis* (Holmes-Davis et al. 2005; Noir et al. 2005) or rice (Dai et al. 2006) did not detect the presence of glycine-rich proteins (GRPs), whereas we identified a number of GRPs (see below). GRPs were also reported in a study on *Arabidopsis* pollen coat proteome (Mayfield et al. 2001), although they were

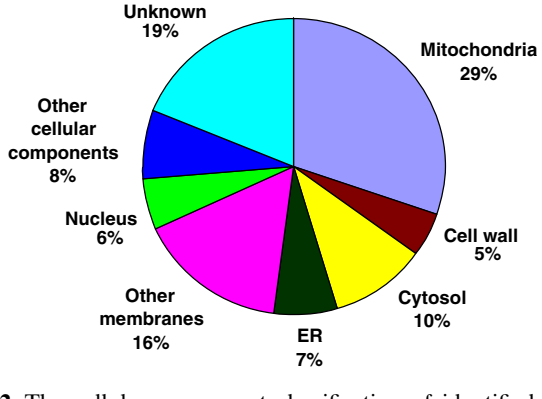

Fig. 2 The cellular component classification of identified *Arabidopsis* pollen proteins using gene ontology database at TAIR (http://www.arabidopsis.org/). The *percentage* values indicate the proportion of total number of proteins within that category

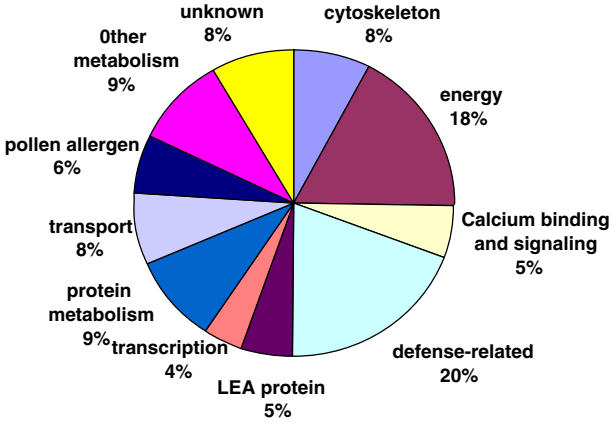

Fig. 3 Functional classification of identified proteins from mature *Arabidopsis* pollen. The *percentage* values indicate the relative proportion of total number of proteins within each group

different from those reported here. Further, about 27% of the polypeptides reported by Noir et al. (2005) have unknown function, whereas the corresponding figure is 8% for our study, as in Holmes-Davis et al. (2005). These discrepancies could be due to a number of factors including different methods of protein extraction and separation, spot selection for analysis, and different ecotypes used. That Ca^{2+}-binding proteins were not found by Holmes-Davis et al. (2005) could be attributed to the low pI of these proteins (4–4.5), which were likely excluded from their gels. In any event, 15% of the proteins involved in general metabolism were common in all three studies.

Cytoskeletal proteins

A number of proteins were identified as actin and profilins (spots 42b, 51, 130, 143, 144, 146, and 149b), which have a role in cytoskeleton and cell organization. A functional actin cytoskeleton is a prerequisite for successful pollen germination and tube growth (Drøbak et al. 2004; Wang et al. 2004). The actin cytoskeleton coordinates the transport of vesicles containing new cell wall materials and plasma membrane to the tip of the growing pollen tube, acts as a track system for cytoplasmic streaming, and appears to be a target for calcium-mediated pollen tube growth (Drøbak et al. 2004; Wang et al. 2004; Cardenas et al. 2005). Profilin is a key actin-binding protein involved in pollen tube growth (Hepler et al. 2001) and its presence has also been reported in mature *Arabidopsis* (Columbia ecotype) (Holmes-Davis et al. 2005; Noir et al. 2005) and rice pollen (Dai et al. 2006). Parallel increases in actin and profilin were reported in developing anthers of rice (Kerim et al. 2003).

Calcium-binding proteins

Several proteins were identified as Ca^{2+}-binding proteins, including calmodulin, calreticulin (CRT), and polcalcin (spots 22, 23, 37, 38, 99, 110, 112, 118, 127, 136, 140, and 141; Fig. 1, Table 1). Calcium plays an important role in pollen germination and tube growth (Malhó et al. 2000). There are complex Ca^{2+} homeostatic mechanisms, including pumps, ion channels, and Ca^{2+} buffers, involved in controlling Ca^{2+} transport (Iwano et al. 2004). Spots 136, 140, and 141 were identified as calmodulin, which appears to work with cyclic nucleotides to regulate the opening of ion channels, and is involved in Ca^{2+} signaling (Bouche et al. 2005) and pollen germination and tube growth (Golovkin and Reddy 2003). Spots 22, 23, 37, and 38 were identified as the high capacity, low affinity Ca^{2+}-binding protein CRT. This is an ER localized protein that is important for ion buffering, and appears to coordinate Ca^{2+} mobilization. CRT was also reported in rice (Dai et al. 2006) and *Arabidopsis* pollen (Noir et al. 2005), and in *Petunia* pollen tubes and styles (Lenartowska, et al. 2002). The presence of CRT in *Arabidopsis* flowers has been demonstrated, with a proposed role in anther maturation and dehiscence (Nelson et al. 1997), in addition to its role in Ca^{2+} binding, and as a molecular chaperone. A putative Ca^{2+}-binding protein (spot 99) and a calmodulin-binding protein (spot 20) have also been identified, and these may have unspecified roles in Ca^{2+} signal transduction in the pollen during germination.

Synaptosomal-associated protein (SNAP-25) was identified (spots 49 and 50) as a plasma membrane associated protein involved in Ca^{2+} transport and the activation and maintenance of store-mediated Ca^{2+} entry (Chieregatti et al. 2004). This protein has a role in vesicle-mediated transport and protein-mediated disease resistance (Collins et al. 2003). The presence of a large number of Ca^{2+}-binding proteins in the mature pollen is in support of their requirement for germination and tube growth.

Defense-related proteins

Defense-related proteins include resistance proteins, defense-regulated proteins, proteins involved in apoptosis, cell rescue, stress response, and detoxification. Many of the plant responses to external factors, e.g., pathogen attack, are related to the production of reactive oxygen species (ROS). Recent evidence suggests that ROS function as cellular second messengers that modulate many different proteins leading to a variety of responses (Foyer and Noctor 2005). However, the excess production of ROS under biotic and abiotic stresses causes oxidative damage to cellular compartments (Apel and Hirt 2004). Plants combat oxidative stress by inducing various protective enzymes and anti-oxidants. In *Arabidopsis* pollen, a number of protein spots involved in detoxification of ROS were identified: i.e., superoxide dismutase (spots 80 and 138), ascorbate peroxidase (68, 70, 87, and 101), dehydro-ascorbate reductase (82 and 91), glutathione transferase (74 and 113), phospholipid hydroperoxide glutathione peroxidase (106 and 117), lipoxygenase (126), and alkyl hydroperoxide reductase (95 and 96a) (Table 1). All these enzymes are known to play a crucial role in oxidative stress and the ascorbate-glutathione cycle in plants (Chew et al. 2003), and their presence in the pollen may provide protection against

oxidative stress. Most of these enzymes were also reported in *Arabidopsis* (Columbia ecotype) (Holmes-Davis et al. 2005; Noir et al. 2005) and rice pollen (Dai et al. 2006).

Several luminal binding proteins (BiP, spots 8, 31, 56, 57, 71, 72, and 107), and protein disulfide isomerases (PDI, spots 13, 21, 46, 53, 58, 59, and 60) belonging to the thioredoxin family, were also identified. These proteins are localized in ER and are known as molecular chaperones involved in proper protein folding, a necessary process for protein transport. Under stress, the folding or assembly of proteins is disordered and this is associated with enhanced expression of genes such as *BiP* and *PDI* (Koizumi et al. 2001). *PDI* has been implicated in fungal pathogen resistance in wheat (Ray et al. 2003). The presence of PDI and BiP in *Arabidopsis* and rice pollen (Dai et al. 2006) may reflect their role in stress tolerance during pollen maturation.

Protein phosphatase 2C, an enzyme that belongs to a class of ubiquitous and evolutionarily conserved serine/threonine protein phosphatases, was identified (spot 109). This enzyme acts as a negative regulator of stress/abscisic acid (ABA) signaling (Zhang et al. 2004). Since ABA is known to play a role in seed and bud dormancy (Zeevaart and Creelman 1988), protein phosphatase 2C may be important for breaking pollen dormancy at the time of germination. Protein spots 131 and 137 were identified as desiccation-related proteins and spot 10 as heat shock protein. Since the pollen grain is an extremely desiccated structure at maturity, these proteins may serve to protect the cytoplasmic contents in the pollen. Desiccation-related proteins were also reported in the *Arabidopsis* Columbia ecotype pollen (Noir et al. 2005).

A number of LEA-like proteins or proteins containing the LEA domain were identified in *Arabidopsis* pollen (spots 39, 52, 75a, 83, 114, 139, and 148). LEA proteins have been shown to accumulate during seed maturation, and their expression has been linked to the acquisition of desiccation tolerance. Many LEA proteins are induced by cold or osmotic stress, or by exogenous ABA, or are expressed constitutively (Wang et al. 2003). Though the precise function of LEA is not known, recent transgenic studies suggest their roles include stress tolerance (Park et al. 2005) and protection of enzyme activity and protein aggregation under water stress (Goyal et al. 2005). LEA-like proteins in *Arabidopsis* pollen may thus provide protection against desiccation during pollen maturation in addition to other unknown functions. LEA-like proteins were also reported in one other study on *Arabidopsis* pollen (Noir et al. 2005) and in lily pollen (Miki-Hirosige et al. 2004), but not in rice pollen (Dai et al. 2006).

Another group of proteins identified in *Arabidopsis* pollen were GRPs (spots 24, 86, 108, 123, 124, and 132; Table 1). Three of these were identified as the RNA-binding proteins AtGRP8 and AtGRP7, and a fourth as the cell wall protein GRP2. GRPs have been implicated in post-transcriptional regulation of gene expression, including RNA processing, which is involved in developmental regulation in plants (Sachetto-Martins et al. 2000). The expression levels of many GRPs have been shown to be up-regulated by various stress conditions, particularly cold and salinity, and they play an important role in defense (Kim et al. 2005). The presence of GRPs in *Arabidopsis* pollen at maturity may, therefore, provide another shield against environmental stresses. A group of lipid-binding GRPs known as oleosins were reported in *Arabidopsis* pollen coat (Mayfield et al. 2001).

Pollen allergens

Pollen grains are known to have a number of proteins which act as allergens (Mohapatra and Knox 1996). Spots 110, 112, and 118 were identified as polcalcins, which are known to act as allergens in a number of species in addition to their role in Ca^{2+}-binding. Other proteins identified in *Arabidopsis* pollen, e.g., profilins, BiP, and calcium binding proteins, also act as allergens (Radauer and Breiteneder 2006).

Energy-related proteins

A number of proteins identified were those involved in energy conversion reactions. These proteins are mainly associated with electron transport in addition to glycolysis, pentose-phosphate-pathway, and TCA-cycle. The pollen grain, like the seed, requires energy during germination and tube growth, and it appears that the mature pollen has this energy machinery in place at the time of maturity. Many studies have shown the presence of enzymes in pollen that participate in energy conversion, general cell maintenance, and metabolism (Holmes-Davis et al. 2005; Noir et al. 2005; Dai et al. 2006). The *Arabidopsis* pollen transcriptome studies have also shown a number of transcripts related to the enzymes involved in energy metabolism (Honys and Twell 2003).

Other proteins

It is known that pollen germination is largely dependent on translation of pre-synthesized mRNAs (Mascarenhas 1990). Therefore, translational control and signal transduction play an important role in pollen germination and tube growth (Honys and Twell 2003).

Spot 125 was identified as translational initiation factor 5A-2 (eIF-5A 2), and spot 129 as 60S ribosomal protein involved in translational elongation. These proteins could, therefore, potentially be important for translation of stored mRNA during pollen germination. The translation initiation factor 5A was identified in rice pollen (Dai et al. 2006), and a putative translation initiation factor transcript is known to be selectively expressed in *Arabidopsis* pollen (Honys and Twell 2003). A number of proteins involved in protein transport within the cell were also identified.

Spot 103b was identified as a proteosome multicatalytic endopeptidase known to eliminate damaged or unneeded proteins and to participate in regulation by targeting and degrading short-lived regulatory proteins (Coux et al. 1996). Spots 111 and 116 were pectin methylesterase (PME) inhibitors, which play an important role in post-translational modification of PME, and were also reported in other studies on *Arabidopsis* and rice pollen (Holmes-Davis et al. 2005; Noir et al. 2005; Dai et al. 2006). PME also has roles in fruit ripening, microsporogenesis, pollen tube growth, seed germination, and hypocotyl elongation (Pilling et al. 2004).

Unknown proteins

Spots 5, 6, 25, 30, 47, 133, 147, and 149a, were identified as hypothetical/unknown or expressed proteins from *Arabidopsis* database, with no well-defined function. Spot 47 contains a clathrin light chain domain, which is a major vesicle coat protein. It is possible that this protein might participate in vesicle formation during pollen tube growth. The identified protein in spot 133 has no corresponding transcript in the database. A large number of transcripts (Honys and Twell 2003) and proteins reported in *Arabidopsis* and rice pollen (Holmes-Davis et al. 2005; Noir et al. 2005; Dai et al. 2006) also belong to the group with unknown function.

Many Arabidopsis pollen proteins are similar to pollen transcripts

Arabidopsis pollen transcriptome studies reported that approximately 10% of transcripts are pollen-specific (Honys and Twell 2003). Our proteome analysis has shown that 12% of the identified proteins were pollen-specific, i.e., actin, profilins, calmodulin, initiation factor 5A, and a hypothetical protein, based upon their corresponding pollen specific transcripts. Thus, in addition to the presence of transcripts, a number of proteins needed for germination and tube growth are in place at the time when pollen is released from the anther. Many of the proteins involved in energy and defense-related mechanisms, including stress related proteins, were also similar to the reported transcripts (Honys and Twell 2003; Pina et al. 2005). However, the frequency of occurrence of abundant proteins has an inverse relationship with the transcript level (Holmes-Davis et al. 2005).

Arabidopsis pollen has a number of proteins similar to those in seeds

The pollen grain at maturity, like the seed, is a dormant, desiccated structure and both are dispersal agents in higher plants. Although the pollen grain is a haploid gametophytic tissue and the seed a diploid sporophytic tissue, it is interesting to note that the pollen has a number of proteins similar to those in seed. A comparison of the pollen proteome with that of seed proteome of *Arabidopsis* (Gallardo et al. 2001), wheat (Vensel et al. 2005), and tomato (Sheoran et al. 2005) indicates that many proteins, e.g., LEA, actin, profilins, dismutase, dihydroascorbate reductase, enolase, PDI, BiP, and ATP synthase, are common to both these structures. LEA proteins likely play a role in stress tolerance during desiccation of pollen and the seed, and actin and profilins would contribute to the formation of the cytoskeleton during germination and growth of both these structures. Other similar proteins and enzymes in the pollen and seed would be required in metabolic processes. One of the striking differences between the two structures was the lack of storage proteins identified in the pollen, e.g., legumins, cruciferin, and vicilins, which are abundant in the seeds. The transcriptome analysis of *Arabidopsis* pollen also revealed the absence of storage protein-related transcripts (Becker et al. 2003; Honys and Twell 2003; Pina et al. 2005). This may be due to the fact that the seed, upon germination, requires stored proteins for extensive meristematic activity to form a young seedling with a root and shoot system. In contrast, pollen germination and tube growth mainly involve extension of the vegetative cell and, therefore, the requirement of stored proteins may be limited.

Conclusions

This study on the proteome analysis of *Arabidopsis* pollen reports on a number of proteins not reported earlier, i.e., calcium binding proteins, GRPs, LEA-like proteins, various isoforms of MDH, transcription factor, initiation factor, protein phosphatase C, and profilin 4. However, many proteins identified were similar to two

other studies on *Arabidopsis* pollen. Our study was on the Landsberg erecta ecotype in contrast to other studies on the Columbia ecotype and this, along with differences in the methods of protein extraction and spot selection, would explain the differences in the pollen proteins between these studies. Nevertheless, together, these three studies on the proteome of two different ecotypes of *Arabidopsis* advance our knowledge on the nature and function of proteins present in the pollen grain at maturity. The proteome of *Arabidopsis* pollen is, however, not yet fully analyzed and further work is needed to identify additional proteins, especially the low abundance proteins, and their roles in pollen.

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