Journal of Experimental Botany, Vol. 58, No. 13, pp. 3525–3535, 2007
doi:10.1093/jxb/erm199 Advance Access publication 5 October, 2007
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Proteomic analysis of tomato (*Lycopersicon esculentum*) pollen

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Received 5 July 2007; Revised 24 July 2007; Accepted 27 July 2007

Abstract

In flowering plants, pollen grains are produced in the anther and released to the external environment with the primary function of delivering sperm cells to the female gametophyte. This study was conducted to identify proteins in tomato pollen and to analyse their roles in relation to pollen function. Tomato is an important crop which is grown worldwide and is an excellent experimental system. Proteins were extracted from pollen, separated by two-dimensional gel electrophoresis (2-DE), and identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and peptide mass fingerprinting. Of the 960 spots observed on Colloidal Coomassie Blue (CCB)-stained 2-DE gels, 190 were selected for analysis. Of these, 158 spots, representing 133 distinct proteins, were identified by searching the NCBInr and Expressed Sequence Tag databases. The identified proteins were classified based on designated functions and the majority included those involved in defence mechanisms, energy conversions, protein synthesis and processing, cytoskeleton formation, Ca^{2+} signalling, and as allergens. A number of proteins in tomato pollen were similar to those reported in the pollen of other species; however, several additional proteins with roles in defence mechanisms, metabolic processes, and hormone signalling were identified. The potential roles of the identified proteins in the survival strategy of the small, independent, two-celled pollen grain of tomato, and subsequently in pollen germination and tube growth are discussed.

Key words: *Lycopersicon esculentum*, MALDI-TOF MS, pollen, proteomics, Solanaceae, tomato, two-dimensional gel electrophoresis.

Introduction

In angiosperms, pollen grains (male gametophytes) are the dispersal agents of sperm cells and are vital for successful sexual reproduction and subsequent seed and fruit production. After release from an anther, pollen grains are carried by insects, wind, or other agents to the stigma of a carpel, where they germinate and deliver sperm cells to the female gametophyte via the formation of pollen tubes. The development of pollen, microsporogenesis and microgametogenesis, involves the co-ordinated expression of several genes in different tissues of an anther (Koltunow *et al*., 1990; McCormick, 2004; Ma, 2005), and pollen grains at maturity contain a large number of transcripts with designated roles in cell wall metabolism, cytoskeleton formation, cell signalling, and vesicle transport (Becker *et al*., 2003; Honys and Twell, 2003, 2004; Pina *et al*., 2005).

Rapid advances in proteomic technologies, along with completion of the *Arabidopsis* and rice genome sequence projects and the availability of comprehensive public sequence databases, have provided tremendous impetus to plant proteomics research (Hirano *et al*., 2004; Rose *et al*., 2004; Rossignol *et al*., 2006). Proteomic analyses of various plant reproductive processes have been conducted, including the identification of sporophytic and gametophytic proteins in normal microspore development (Kerim *et al*., 2003; Miki-Hirosige *et al*., 2004), changes in anther proteins due to cold stress (Imin *et al*., 2006), proteins in relation to pollen germination and tube growth (Dai *et al*., 2006, 2007), and self-incompatibility (Kalinowski *et al*., 2002). The proteome analyses of mature *Arabidopsis* and rice pollen have also been conducted, and many proteins identified correspond to the known transcripts in pollen, in addition to several other proteins in each of these studies

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(Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Dai *et al*., 2006; Sheoran *et al*., 2006).

The objective of this study was to analyse the proteome of tomato pollen. Tomato is an important crop grown around the world (Rick, 1980) and is also known to have significant effects on human health (Willcox *et al*., 2003; Omoni and Aluko, 2005). Tomato pollen grains are bi-cellular with a large vegetative cell and a small generative cell; the latter divides to form two sperm cells during pollen germination and tube growth, unlike the *Arabidopsis* and rice pollen which are tri-cellular. Pollen development in tomato has been studied extensively at both the light microscope and ultrastructural levels (Sawhney and Bhadula, 1988; Polowick and Sawhney, 1993*a*, *b*), and a number of male-sterile mutants are available in tomato which makes it an excellent model system for genetic and molecular investigations on pollen development, and in hybrid seed programmes (Sawhney, 1994; Gorman and McCormick, 1997).

By combining two-dimensional gel electrophoresis (2-DE) with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), and by using the available databases for tomato and other species, as well as tomato expressed sequence tags (ESTs), a comprehensive analysis of the tomato pollen proteome has been performed. Many of the proteins identified in this study have designated roles in defence mechanisms, energy conversion, pollen germination, and pollen tube growth, and some possibly in sperm cell formation. To our knowledge, this is the first proteomic study on tomato pollen, and several of the proteins reported here have not been identified in the pollen of other species.

Materials and methods

Plant growth

Seeds of tomato (cv. Rutgers) were germinated in 16 cm plastic pots containing Tera-lite Redi-earth mix. Young seedlings and plants were subsequently grown in a growth chamber at 26/23 °C (day/night) and under 16/8 h light/dark conditions. Illumination was provided by fluorescent tubes (F72T12/CW/VHO; Sylvania, USA) and incandescent bulbs at a photon flux density of 100–200 μmol m^{-2} s^{-1}.

Pollen collection

Pollen grains were collected from freshly open flowers by shaking the anthers on a glass slide, checked under a dissecting microscope, and any debris removed with a needle. Pollen samples were pooled in an Eppendorf tube and the purity of pollen was again determined under a light microscope. Each pooled sample represented pollen from approximately 400 flowers. Pollen was either used immediately or stored at –80 °C until further use. The viability of each pollen sample was tested using an *in vitro* germination test (Shivanna and Sawhney, 1995) and pollen germination was in the range of 70–75%. Three separate batches of pooled pollen samples were used for protein extraction.

Protein extraction

The pollen samples (~50 mg each, collected from 150–200 flowers) were ground to a fine powder in a pestle and mortar in liquid nitrogen, and extracted with acetone containing 10% TCA and 1% DTT. The samples were kept at –20 °C for 2 h and centrifuged at 25 000 *g* for 20 min at 4 °C. The resulting pellet was washed by suspending in acetone containing 1% DTT, incubated at –20 °C for 2 h, and centrifuged; it was re-suspended in acetone, sonicated (3×15 s), and centrifuged at 25 000 *g*. The pellet was vacuum-dried and then dissolved in urea 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 collected. The resulting pellets were re-extracted with urea buffer and the supernatant combined with that collected earlier. The resulting protein samples were centrifuged again for 20 min at 25 000 *g*, quantified (3–3.5 mg per 50 mg pollen) using the Bio-Rad DC protein Assay Kit (Bio-Rad, Hercules, CA, USA), and either used immediately or stored at –80 °C for later use.

Two-dimensional gel electrophoresis (2-DE)

2-DE was carried out as previously described (Sheoran *et al*., 2005, 2006). Isoelectric focusing (IEF) was performed using the Multiphor II horizontal electrophoresis system (Amersham Biosciences, Uppsala, Sweden) and 18 cm Immobiline Dry Strips of 4–7 or 3–10 linear pH gradients (Bio-Rad, Hercules, CA, USA). 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 3500 V over 2 h, and holding at 3500 V until a total of 90 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 20 mM 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 15 mA gel^{-1}, and then at 20 mA gel^{-1} until the dye front reached the bottom of the gel, in an SDS electrophoresis buffer containing 25 mM TRIS base, 192 mM glycine, and 0.1% SDS, pH 8.3 in a PROTEAN II XL multi-cell (Bio-Rad, USA).

Gel staining and image analysis

Gels were fixed overnight in 50% (v/v) ethanol with 10% (v/v) orthophosphoric acid, washed with water (3×20 min), and stained with Colloidal Coomassie Blue G-250 (CCB) as described earlier (Sheoran *et al*., 2006). After washing with water, gels were scanned, annotated, and analysed for spot number using Phoretix 2D Image analysis software (UBI, Canada). Two replicate gels (for both pH 4–7 and 3–10) were run for each of three different pooled pollen samples collected from different batches of plants.

Mass spectrometry and protein identification

Of the spots observed consistently on CCB-stained 2-DE gels, 190 were selected for mass spectrometric analysis from both pH 4–7 and 3–10 gels, as previously described (Sheoran *et al*., 2005, 2006). Excised protein spots were automatically de-stained, dehydrated, reduced with DTT, alkylated with iodoacetamide, and digested with trypsin using a MassPREP protein digest station (Micromass, Manchester, UK) according to the recommended procedure. The resulting tryptic digests were concentrated and desalted using C_{18}

ZipTips (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's instructions. Samples were then analysed by MALDI-TOF MS on a Voyager-DE STR instrument (Applied Biosystems, Framingham MA, USA) operating in the positive ion and reflectron modes as described earlier (Sheoran *et al*., 2005, 2006). Spectra were acquired in the 700–3000 *m*/*z* range, processed with Mascot Distiller 2.0.0 (www.matrixscience.com), and the resulting peak lists used to identify the corresponding proteins in NCBInr (non-redundant) and Swiss-Prot databases by peptide mass fingerprinting (PMF) using the Mascot (www.matrixscience.com) search engine. Searches were performed using the following parameters: trypsin as the proteolytic enzyme, allowing for one missed cleavage; carbamidomethylation of cysteine as a fixed modification; oxidation of methionine as a variable modification. Proteins identified with a Mowse score greater than 66 (significant at 95% confidence interval) are reported. Because of the limited availability of tomato protein sequence information, database searches were also performed using the NCBI tomato EST database.

Results and discussion

The soluble proteins extracted from mature tomato pollen, separated by 2-DE on pH 4–7 and 3–10 IPG strips, and stained with CCB are shown in Fig. 1A and B, respectively. A total of 960 reproducible protein spots were detected using the pH 4–7 IPG strips (Fig. 1A) and 870 using the pH 3–10 strips (Fig. 1B), indicating better resolution on pH 4–7 gels, as previously observed for rice pollen (Dai *et al*., 2006). The number of protein spots observed in this study is comparable with that of our proteome analysis of *Arabidopsis* pollen (Sheoran *et al*., 2006) and is substantially higher than that reported in two other studies on *Arabidopsis* pollen (Holmes-Davis *et al*., 2005; Noir *et al*., 2005).

One hundred and ninety protein spots were selected throughout the molecular mass and isoelectric point (pI) ranges of pH 4–7 and 3–10 gels and analysed by MALDI-TOF MS. Of these, 158 spots representing 133 distinct proteins were successfully analysed. Despite the limited availability of tomato protein sequence data, it was possible to identify 83% of the selected spots. This was achieved by concentrating and desalting the tryptic digests using C_{18} ZipTips, and by processing the MS data with Mascot Distiller 2.0.0. The identified proteins, along with the gene index (gi) number and Mowse score, are listed in Table 1.

Some of the identified proteins were present as multiple spots on 2-DE gels. These may correspond to multiple isoforms, which could play an important role in pollen development and germination by diversifying the functions of proteins in the haploid genome. Multiple spots corresponding to the same protein have been reported in other proteomic studies (Kerim *et al*., 2003; Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Dai *et al*., 2006; Sheoran *et al*., 2006).

The predicted molecular masses and pIs for the majority of the identified proteins were generally consistent with the experimental data, as judged from the location of spots on 2-D gels; however, there were some exceptions. For example, spots 31, 47a, 65, 111, and 113 had an apparent molecular mass greater than the corresponding identified protein, whereas spots 5, 20, 24, 58, and 145 had a molecular mass lower than the predicted value. These deviations in molecular mass and pI, as well as multiple spots for the same protein, could be due to various factors, including post-translational modifications, protein degradation, and partial synthesis of proteins during pollen maturation, protein translation from alternatively spliced mRNAs, or protein homologues that may be unique to pollen (Sheoran *et al*., 2006).

Functional grouping of identified proteins

The identified proteins were categorized into 12 functional groups (Fig. 2) based on predicted protein function and defined criteria (Berardini *et al*., 2004; Sheoran *et al*., 2006). More than half of the identified proteins were in three major groups, i.e. energy (19%), defence-related (18%), and protein synthesis and processing (18%). The other groups included proteins involved in cytoskeleton (cell biogenesis and organization), membrane transport, hormone metabolism and signalling, Ca^{2+} binding and signalling, pollen allergens, other metabolism, and those of unknown function (Fig. 2). Proteins associated with hormone metabolism and signalling, Ca^{2+} binding and signalling, pollen allergens, and glycine-rich proteins (GRPs) were grouped separately in Fig. 2 because of their established roles in pollen function. In *Arabidopsis* and rice pollen also, proteins belonging to most, but not all, of these groups were identified and the majority of them were associated with energy, defence, and protein synthesis and processing (Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Dai *et al*., 2006; Sheoran *et al*., 2006). For comparison purposes, proteins in tomato pollen which are common with *Arabidopsis* and/or rice pollen are indicated in the last column in Table 1.

Each group of proteins in tomato pollen and their potential roles in pollen function are discussed separately below.

Defence-related proteins

Proteins included in this group are associated with both biotic and abiotic stresses. The biotic stress-related proteins included the Pto-disease resistance protein, Pto-like serine/threonine kinase, I2 (disease resistance protein), tomato mosaic viral (ToMV) coat protein, and a hypothetical protein PGEC 13.19. Plants expressing the *Pto* gene encoding a serine/threonine kinase protein were shown to be resistant to both bacterial and fungal pathogens (Tang *et al*., 1999; Pedley and Martin, 2003), and the *Pto*-mediated resistance has been used to control the bacterial speck disease in different tomato cultivars

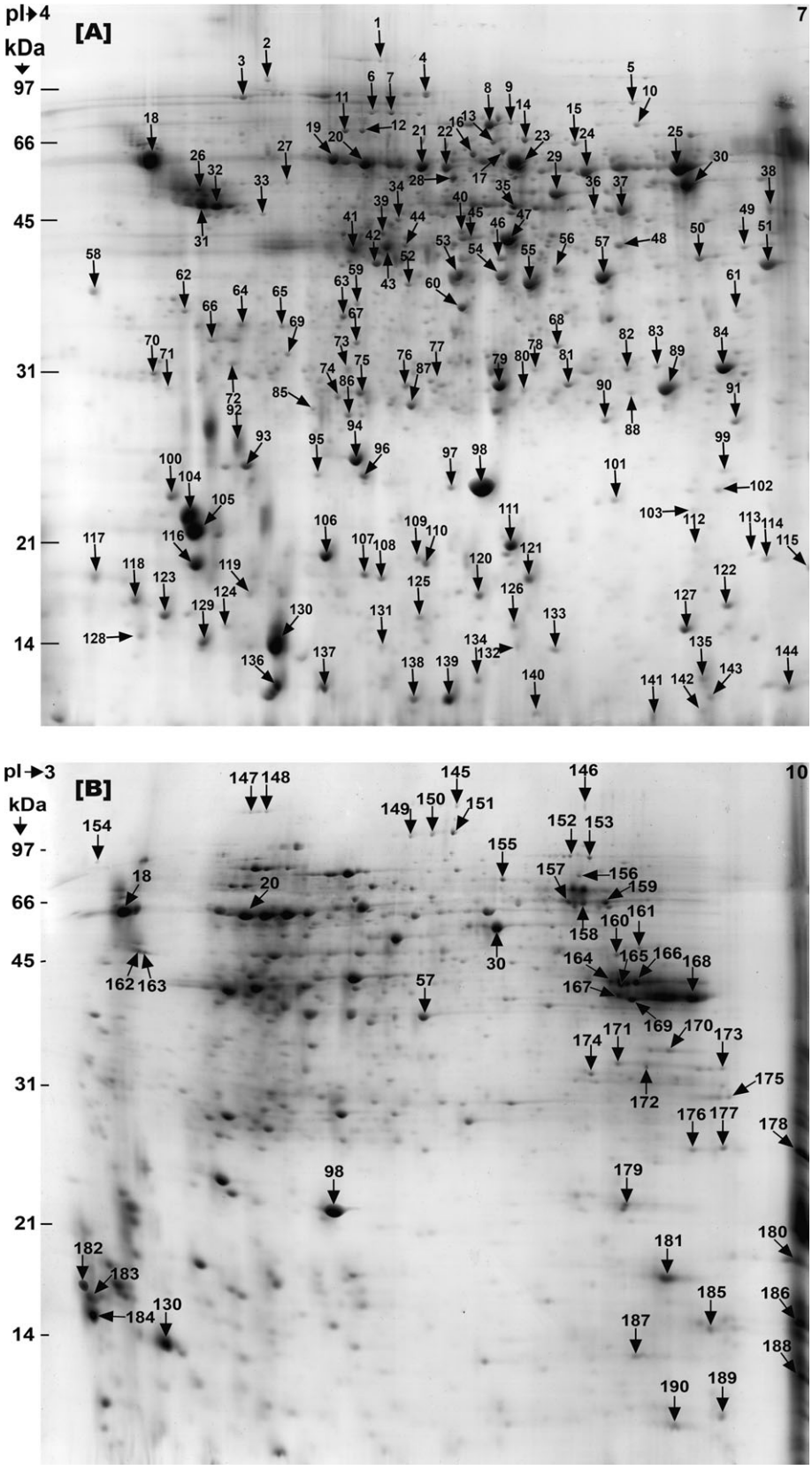

Fig. 1. Colloidal Coomassie Blue-stained 2-DE gels of tomato mature pollen protein extract (600 μg). (A) Proteins separated on pH 4–7 IPG strips. (B) Proteins separated on pH 3–10 NL IPG strips. Molecular masses (kDa) are shown on the left and pI ranges at the top corners of each figure. The numbered spots were analysed by MALDI-TOF MS, and the identified proteins listed in Table 1.

Table 1. *Tomato (*Lycopersicon esculentum*) pollen proteins separated by 2-DE (Fig. 1A, B) and identified using MALDI-TOF-MS*

The proteins are listed under broad functional categories; however, many of the proteins have more than one function.

Spot no.	Gene index (gi)	Species[a]	Protein identity	Mw/pI[b]	NPM/Cov.[c] (%)	Mowse score[d]	Proteins in pollen of other species[e]
Defence-/stress-related proteins							
7	300265	LE	HSP68=68 kDa heat stress DnaK homologue	62.5/5.2	10/21	85	
10	7671443	AT	Cytochrome P450-like protein	64.6/6.2	11/31	91	
22	1532049	SO	Monodehydroascorbate reductase	54.0/6.7	14/30	124	AT
24	5759320	LE	Copper/zinc superoxide dismutase	32.8/6.5	13/25	176	AT, OS
38	444340	AT	Catalase	57.2/6.6	24/34	211	AT
41*	5891529	LE	EST276332 (callus) similar to gi15236375 Serine hydroxymethyltransferase 4	52.0/6.8	7/23	94	
56	50252724	OS	Putative glutathione transferase	37.7/5.9	14/38	130	AT, OS
62	12231300	LE	Ripening-regulated protein DDTFR10	22.2/4.7	9/38	99	
68	21039134	LE	Ascorbate peroxidase	42.4/8.9	17/41	144	AT, OS
78	62526498	LE	Ascorbate peroxidase	27.0/5.9	16/42	140	AT, OS
81	70913175	LP	Pto-disease resistance protein	34.7/5.4	10/44	110	
82	73761753	LE	Cytosolic ascorbate peroxidase 2	27.5/6.0	13/42	153	AT, OS
90	62526498	LE	Ascorbate peroxidase	27.6/5.9	14/47	127	AT, OS
93	77641257	ST	I2 (disease resistance protein)	25.3/5.3	6/32	71	
95	30841938	LE	Thioredoxin peroxidase 1	17.5/5.2	7/36	91	
96	30841938	LE	Thioredoxin peroxidase 1	17.5/5.2	13/60	124	
104	28170732	LE	Coat protein (ToMV)	17.9/4.9	7/55	89	
105	229181	LE	Coat protein (ToMV)	17.7/4.9	7/54	93	
118	115465191	OS	Putative group3 LEA protein	20.5/5.9	11/51	101	AT
119	15778360	LE	Coat protein (ToMV)	17.9/4.9	6/52	123	
120	854248	LE	Cytosolic Cu, Zn superoxide dismutase	15.3/5.6	5/35	76	AT, OS
134	15054759	SS	Putative Pto-like serine/threonine kinase	14.6/6.8	6/51	85	
135	15054741	SB	Pto-like serine/threonine kinase	19.7/6.1	10/60	111	
142	3850778	LE	Glutaredoxin	11.5/8.8	6/57	79	AT
158	438247	ST	Glycine hydroxymethyltransferase	57.2/8.5	26/46	209	
180	54261837	ST	Hypothetical protein PGEC13.19	20.0/9.2	8/50		103
Energy-related							
4	758340	ST	76 kDa mitochondrial complex I subunit	81.0/5.9	13/23	93	
8	4582924	ST	Phosphoglycerate mutase	61.0/5.4	19/34	134	AT, OS
9	4582924	ST	Phosphoglycerate mutase	61.0/5.4	12/21	78	AT, OS
14	410634	ST	Cytochrome *c* reductase-processing peptide	59.5/6.3	16/31	89	
15	10444388	ST	Dihydrolipoamide dehydrogenase precursor	53.4/6.4	14/26	93	OS
19	19685	NP	ATP synthase beta subunit	59.9/5.9	20/43	141	AT, OS
21	8415909	AT	ATP binding/H-exporting ATPase	59.8/6.2	18/39	154	AT, OS
23	19281	LE	Enolase	48.0/5.7	12/36	121	AT, OS
33	56784992	OS	ATP synthase beta subunit	45.2/5.3	15/34	101	OS
34	20465305	AT	Putative hexokinase	54.5/5.5	13/22	88	OS
42*	6533434	LE	EST298561 (leaf) similar to gi3850999 pyruvate dehydrogenase E1 beta subunit isoform I (*Zea mays*)				
48	52139816	LE	Mitochondrial MDH	36.2/8.7	15/57	143	AT, OS
51	21388550	ST	Mitochondrial MDH	36.4/8.5	12/43	128	AT, OS
52	19281	LE	Enolase	48.0/5.7	18/37	136	AT, OS
53	75221385	LE	Fructokinase-2	35.0/5.8	14/39	110	OS
54	75221385	LE	Fructokinase-2	35.0/5.8	20/57	193	OS
58	37991922	OS	Cytochrome *c* oxidase subunit 6b-1	19.1/4.3	6/41	94	
59	1161573	LE	Enolase	35.3/6.3	19/50	205	AT, OS
60	1161573	LE	Enolase	35.3/6.3	11/36	122	AT, OS
61	21388550	ST	Mitochondrial MDH	36.4/8.5	11/33	122	AT, OS
63	19281	LE	Enolase	48.0/5.7	10/39	103	AT, OS
65	19281	LE	Enolase	48.0/5.7	11/28	112	AT, OS
67	1161573	LE	Enolase	35.3/6.3	15/44	205	AT, OS
85	1915974	LE	Fructokinase	35.0/5.8	11/38	140	OS
86	1915974	LE	Fructokinase	35.0/5.8	13/48	156	OS
89	38112662	SC	Triose phosphate isomerase cytosolic isoform	27.0/5.7	11/37	103	AT, OS
94	48209968	SD	Mitochondrial ATP synthase D-chain	19.8/5.3	18/64	186	AT, OS
111	1915974	LE	Fructokinase (fragment)	35.0/5.8	8/30	139	
129*	58236622	LE	EST/BP893151 (fruit) similar to gi82623399 cytochrome *c* oxidase family protein-like				
133	50916028	LE	Putative vacuolar ATP synthase subunit F	14.4/5.6	8/55		120 AT, OS
140	21360507	AT	Cytochrome *c* oxidase	09.5/5.3	6/45	87	
155	23321340	LE	Dihydrolipoamide dehydrogenase precursor	53.1/6.9	15/24	123	AT, OS
165	8328399	ST	Fructose-bisphosphate aldolase-like protein	39.0/7.5	17/47	216	AT, OS
166	77745438	ST	Unknown (fructose-bisphosphate aldolase)	40.0/8.3	14/44	135	AT, OS

Table 1. (*Continued*)

Spot no.	Gene index (gi)	Species[a]	Protein identity	Mw/pI[b]	NPM/Cov.[c] (%)	Mowse score[d]	Proteins in pollen of other species[e]
167	312179	ZM	Glyceraldehyde 3-P dehydrogenase	36.6/6.4	8/24	75	AT, OS
168	3059140	PS	NAD-dependent G3PDH	39.3/9.0	14/34	131	AT, OS
169	52139818	LE	Cytosolic MDH	36.2/6.5	11/25	129	AT, OS
178*	5273558	LE	EST256617 (leaf) similar to ATP-synthase delta chain oligomycin sensitivity conferral protein (gi4774163)	27.2/9.6	21/67	187	
Protein synthesis and processing							
1	18390588	AT	Cell division cycle protein 48-related	134.5/5.6	16/14	86	AT, OS
2	1346172	LE	Luminal-binding protein precursor	73.5/5.1	18/25	143	AT, OS
3	2654208	SO	Heat shock70 protein	76.3/5.2	15/23	159	AT, OS
5	587564	ST	Mitochondrial processing peptidase-like	59.5/6.2	11/18	95	OS
11	16221	AT	Chaperonin hsp60	61.7/5.2	18/32	110	AT, OS
12	12546	C	Chaperonin 60	61.5/6.3	21/39	140	AT, OS
13	587566	ST	Mitochondrial processing peptidase-like	60.1/6.2	10/28	76	OS
16	82621176	ST	Mitochondrial processing peptidase-like	58.2/5.8	6/18	76	OS
17	82621176	ST	Mitochondrial processing peptidase-like	58.2/5.8	12/22	89	OS
28	7268689	AT	Protein kinase-like protein	48.2/5.8	11/25	89	
31	30025966	NT	Heat shock protein 70	71.3/5.2	19/23	130	AT, OS
44	21493	ST	Mitochondrial processing peptidase	55.0/5.7	12/26	105	OS
66	48209911	ST	Putative elongation factor 1-beta	24.6/4.6	12/50	143	OS
71	19805	NT	Luminal-binding protein	32.1/4.6	10/28	91	
72	729617	NT	78 kDa glucose-regulated protein homologue 1	32.1/4.6	15/43	134	
76	77416969	ST	Unknown (proteosome alpha type-2)	25.6/5.4	7/48	101	
77	77999303	ST	Proteasome-like protein alpha subunit-4	27.3/5.6	12/41	131	
83	4539545	NT	(PRCI) Proteasome subunit alpha type 6	27.0/5.9	11/38	115	OS
100	49425163	LE	Translationally controlled tumour protein-like	19.0/4.6	7/32	77	AT, OS
107	15778156	NT	14-3-3 protein	23.0/5.6	7/35	88	AT, OS
112	78191460	ST	Ubiquitin-conjugating enzyme	16.7/6.2	8/54	88	OS
138	6671194	LE	Cystatin	10.4/5.8	6/67	88	
145	29893543	AT	Putative elongation factor	74.7/6.9	15/24	119	AT
156	29893543	AT	Putative elongation factor	74.7/6.9	15/24	119	AT
162	3986110	SG	Heat shock protein 70 cognate	45.6/5.2	18/41	149	
181	118103	LE	Peptidylprolyl isomerase (PPI) (cyclophilin)	18.2/8.8	14/83	165	AT
Ca^{2+} binding and signalling							
18	1419088	NP	Calreticulin	47.7/4.4	15/26	138	AT, OS
50	7960742	AT	Calcium-binding protein (annexin 7)	36.6/6.4	17/45	172	
106	115447273	OS	Temperature stress-induced lipocalin	22.3/5.2	10/47	87	
124	48209896	SD	Putative calmodulin	16.6/4.9	9/62	113	AT
183	2388889	LE	Calmodulin	13.3/4.1	5/23	87	AT
184	228408	AT	Calmodulin-1	15.5/4.2	8/40	99	AT
Cytoskeleton (cell organization and biogenesis)							
20	2499814	LE	Profilin 1	14.5/5.0	9/40	99	AT, OS
25	32527831	PT	UDP-glucose pyrophosphorylase	52.0/5.7	12/25	88	AT, OS
40	48478827	LE	UDP-glucose: protein transglucosylase-like	41.6/5.8	15/34	142	
43	38194918	PV	Reversibly glycosylated protein	40.7/5.8	9/ 33	94	AT, OS
45	21594350	AT	dTDP-glucose 4-6-dehydratase	44.2/5.6	10/29	82	AT, OS
4	21599	ST	UTP-glucose-1-phosphate uridylyltransferase	52.0/5.4	12/19	84	AT
73	50355625	UP	Actin	42.0/5.2	12/43	112	AT, OS
113	15229001	AT	Pectin methylesterase inhibitor	39.1/6.3	9/23	90	AT, OS
125	1399496	LE	Profilin	14.5/5.0	8/57	107	AT, OS
130	2499814	LE	Profilin-1	14.5/5.0	8/ 40	87	AT, OS
131	2499814	LE	Profilin-1	14.5/5.0	5/33	67	AT, OS
136	7441438	LE	Profilin-1	14.5/5.0	7/52	83	AT, OS
152	15667247	LE	Pectin methylesterase	64.0/9.3	15/20	125	AT, OS
157	5931765	NT	Phragmoplastin	68.5/7.7	18/23	100	
Hormone metabolism and signalling							
26	429108	LE	*S*-adenosyl-L-methionine synthetase	42.6/5.8	10/24	96	AT, OS
29	429108	LE	*S*-adenosyl-L-methionine synthetase	42.6/5.8	13/41	114	AT, OS
30	1084408	LE	*S*-adenosyl-L-methionine synthetase	43.0/5.8	16/39	148	AT, OS
36	15225278	AT	GPA1 (G protein alpha subunit1)	44.9/6.0	17/47	137	
69	52353464	OS	Aminocyclopropane-1-carboxylate oxidase	34.7/5.1	10/22	68	
114	15218243	AT	IAA5; transcription factor	18.7/6.4	9/53	92	
187	15226486	AT	Auxin-responsive calmodulin binding	11.9/8.8	8/52	87	
Glycine-rich proteins							
97*	58247573	LE	EST/BP904102 (leaf) similar to GRP-2				
108	82623423	ST	Glycine-rich RNA-binding protein	17.6/5.6	7/61	154	AT

Table 1. (*Continued*)

Spot no.	Gene index (gi)	Species[a]	Protein identity	Mw/pI[b]	NPM/Cov.[c] (%)	Mowse score[d]	Proteins in pollen of other species[e]
121	799015	ST	Putative glycine-rich RNA-binding protein	17.6/5.6	7/39	124	AT
141	8272390	PP	Glycine-rich protein	10.7/6.3	4/23	69	AT
Nucleic acid metabolism							
55	15229589	AT	Nucleotide-binding protein	36.2/6.7	8/22	83	
57	15229589	AT	Nucleotide-binding protein	36.2/6.7	14/27	126	
87	15230956	AT	DNA binding (MAD2)	23.9/4.8	12/40	130	
127	575953	LE	Nucleotide diphosphate kinase	15.5/6.8	6/44	93	
147	6681343	AT	Putative transitional endoplasmic ATPase	90.1/5.1	19/25	161	
148	18414193	AT	ATP binding/ATPase/CDC48	90.0/5.1	17/24	136	
185	998712	SO	Nucleotide diphosphate kinase type III	17.1/8.1	11/54	116	
Other metabolism							
37	30687061	AT	ATP binding/protein kinase	58.3/6.1	13/26	97	
39	27803873	LE	Succinyl CoA ligase beta subunit	44.8/5.9	18/48	132	AT
46	1419094	NT	Glutamine synthetase	39.4/5.4	11/32	103	OS
47a	2243118	BJ	Glutathione synthetase	60.0/6.6	15/29	116	
47b	1419094	NT	Glutamine synthetase	39.4/5.4	8/17	66	OS
64	29169309	LE	Biotin carboxylase carrier protein	30.4/9.0	11/47	103	
70	75280142	LE	Aspartate carbamoyltransferase (fragment)	30.0/6.0	10/43	114	
79*	7337866	LE	EST313668 (radicle) tomato similar to gi30690243 uridylate kinase	23.0/5.6	10/39	113	
88	11994278	AT	Cysteine synthase	32.4/5.8	13/41	126	
103*	5276857	LE	EST259695 (leaf) similar to gi18399910 3-hydroxyacyl-acyl-carrier protein dehydratase				
149	8439545	ST	Methionine synthase	84.9/5.9	16/19	132	AT
150	8439545	ST	Methionine synthase	84.9/5.9	17/24	141	AT
151	8439545	ST	Methionine synthase	84.9/5.9	19/22	146	AT
160	438254	ST	Aminomethyltransferase (T-protein)	45.0/9.1	15/43	140	AT
161	21537268	AT	Putative acetyl-CoA acyltransferase	47.2/9.0	17/40	153	AT
Membrane transport							
6	27883932	LE	Vacuolar H^+-ATPase A1 subunit isoform	68.8/5.2	13/24	78	AT, OS
75	534916	ST	Soluble inorganic pyrophosphatase	24.4/5.6	10/27	114	AT, OS
91	51854284	OS	GTP-binding protein	27.2/6.4	8/39	66	
170	515358	ST	36 kDa porin I	29.4/7.8	12/39	132	
171	30689271	AT	Unknown protein (Fascin domain)	34.5/7.0	15/44	114	
172	516166	ST	34 kDa porin	30.0/8.9	9/37	103	
173	515360	ST	36 kDa porin II	29.4/7.8	9/29	102	
175	77999247	ST	POM30-like protein (Porin)	29.3/8.9	10/38	98	
Unknown function							
74	21593492	AT	Unknown protein (mitochondrial glycoprotein)	25.0/6.0	7/60	145	
84	15219092	AT	Protein binding/unknown	38.1/5.8	12/30	94	
116	295812	LE	Anther-specific protein LAT 52	17.7/4.9	5/56	83	
122	47496869	OS	Hypothetical protein	18.8/6.6	8/51	100	
153	9755453	AT	Hypothetical protein	93.9/9.5	15/11	98	
159	17978713	AT	Unknown protein	58.0/9.3	16/24	115	
163*	9292405	LE	EST355772 (flower bud at anthesis), no homology with any protein				
179	30678969	AT	Unknown protein	22.3/10.0	11/37	84	

[a] Abbreviations used for various species: AT, *Arabidopsis thaliana*; C, *Cucurbita* Sp; LE, *Lycopersicon esculentum* LP, *Lycopersicon peruvianum*; NP, *Nicotiana plumbaginifolia*; **NT**, *Nicotiana tabacum*; OS, *Oryza sativa*; PP, *Pyrus pyrifolia*; PS, *Pinus sylvestris*; PV, *Phaseolus vulgaris*; SB, *Solanum berthaultii*; SC, *Solanum chacoense*; SG, *Salix gilgiana*; SO, *Spinacia oleracea*; SS, *Solanum sucrense*; ST, *Solanum tuberosum*; UP, *Ulva pertusa*; ZM, *Zea mays*.

[b] Theoretical molecular weight and pI of the identified proteins.

[c] NPM, number of peptides matched and percentage of protein sequence coverage.

[d] Probability-based protein scores greater than 66 are considered significant ($P < 0.05$).

[e] AT, *Arabidopis thaliana*; OS**,** *Oryza sativa*.

* Identified using the EST database. No significant matches were found for the spot numbers not listed in the table.

(Martin, *et al*., 2003). The presence of ToMV coat protein in tomato pollen could provide resistance to viruses, since the constitutive expression of viral coat protein genes is known to be effective in producing virus-resistant plants (Koo *et al*., 2004). The Pto-like serine/threonine kinase and ToMV coat protein were not reported in rice and *Arabidopsis* pollen (Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Dai *et al*., 2006) although they were present in the embryo and endosperm of tomato seed (Sheoran *et al*., 2005).

Pollen grains are free-floating structures and are subject to various abiotic stresses, including drought and extreme

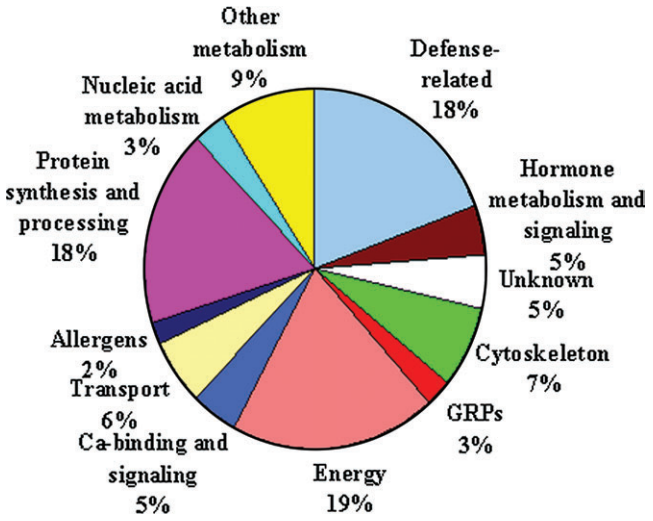

Fig. 2. Functional categorization of proteins identified from mature tomato pollen.

temperatures. Plants produce reactive oxygen species (ROS) in responses to abiotic and biotic stresses. Although ROS are known to serve as second messengers in many developmental processes (Foyer and Noctor, 2005), the excessive production of ROS causes oxidative damage to cellular components (Apel and Hirt, 2004; Gechev *et al*., 2006). Plants have evolved a strategy to combat the ROS by inducing various protective enzymes. Many enzymes known to play a role in the detoxification of ROS were identified in tomato pollen, including superoxide dismutase, thioredoxin peroxidase, ascorbate peroxidase, mono-dehydroascorbate reductase, glutathione transferase, glutaredoxin, and catalase (Table 1). These proteins were also reported in rice and *Arabidopsis* pollen (Table 1; Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Dai *et al*., 2006; Sheoran *et al*., 2006).

Heat shock proteins (HSPs) and luminal-binding proteins play key roles in defence mechanisms, in addition to their roles as molecular chaperones in protein processing. In tomato pollen, HSP 60, HSP 70, HSP 70-cognate, chaperonin 60, and luminal-binding proteins were identified. HSPs are known to act as protectants of protein function (Vierling, 1991; Wang *et al*., 2004) and their accumulation in response to heat stress has been reported in developing pollen (Mascarenhas and Crone, 1996). Other proteins present in tomato pollen, such as temperature stress-induced lipocalin, ripening-regulated protein DDTFR10, and the heat stress DnaK homologue, might also have a role in combating abiotic stresses. One spot representing LEA proteins, which are known to have a role in desiccation tolerance (Park *et al*., 2005), was identified in tomato pollen, whereas 4–7 LEA protein spots were observed in *Arabidopsis* pollen (Noir *et al*., 2005; Sheoran *et al*., 2006).

The defence-related proteins identified in tomato pollen could be part of the survival strategy of these small two-celled structures, which are independent of the parental tissues and, therefore, particularly susceptible to biotic and abiotic stresses.

Energy-related proteins

The presence of a high percentage (19%) of proteins related to energy metabolism correlates well with the large number of mitochondria observed in mature tomato pollen (Polowick and Sawhney, 1993*b*). These include proteins associated with glycolysis, for example phosphoglucomutase, glyceraldehyde 3-P dehydrogenase, triose phosphate isomerase, enolase, and fructokinase; with the TCA cycle, for example MDH, succinyl CoA ligase, dihydrolipoamide dehydrogenase; and with the electron transport chain, for example cytochrome *c* oxidase and reductase, and various subunits of ATP synthase (Table 1). Cytochrome *c* oxidase and reductase were identified only in tomato pollen, and a relatively high number of spots was observed for fructokinase and enolase in tomato compared with *Arabidopsis* and rice pollen. However, most of the energy-related proteins in tomato were also reported in rice and *Arabidopsis* pollen (Table 1; Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Dai *et al*., 2006; Sheoran *et al*., 2006). Pollen germination and tube growth are high-energy-requiring processes and it seems that most of the proteins required for these events are in place in mature tomato pollen. Although the transcriptome of tomato pollen is not yet available, in *Arabidopsis* pollen, the transcripts of energy-related proteins are under-represented (Becker *et al*., 2003; Honys and Twell, 2003, 2004; Pina *et al*., 2005). Indeed, Holmes-Davis *et al*. (2005) showed an inverse relationship of high abundance energy-related proteins between and the corresponding mRNA in *Arabidopsis* pollen.

Protein synthesis and processing

Three spots corresponding to a putative elongation factor and elongation factor-1β involved in protein synthesis were identified in tomato pollen. Proteins involved in proper protein folding, assembly, and localization, including chaperonin 60, cyclophilin, HSPs, luminal-binding proteins, 78 kDa glucose-regulated protein homologue 1, and HSP68 heat stress DnaK homologue, were also identified (Table 1). In addition, a large number of proteins involved in protein degradation such as cystatin, transitional endoplasmic ATPase, mitochondrial processing peptidase-like, proteasome subunits, ubiquitin-conjugating enzyme, cell division cycle protein 48-related, and translationally controlled tumour protein-like were identified (Table 1). There were relatively few proteins involved in protein synthesis compared with those in protein processing and degradation in tomato pollen, as in pollen of other species (Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Dai *et al*., 2006; Sheoran *et al*., 2006). This is consistent with the relatively small number of polysomes

observed in mature tomato pollen (Polowick and Sawhney, 1993*b*). However, the mature pollen has abundant stored mRNA (Schrauwen *et al*., 1990; Honys *et al*., 2000) and translational apparatus (Mascarenhas, 1989), indicating rapid protein synthesis at the onset of pollen germination and tube growth.

Cell biogenesis and organization

Actin cytoskeleton is an essential component of pollen tube growth as it transports new cell wall materials to the growing tip region (Drobak *et al*., 2004). Actin was identified in tomato pollen (Table 1), as in the pollen of other species (Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Dai *et al*., 2006; Sheoran *et al*., 2006). In addition, profilin, a major actin-binding protein involved in pollen tube growth (Taylor and Hepler, 1997), was also identified in tomato pollen (Table 1).

Cell wall loosening and synthesis are essential for pollen germination and rapid pollen tube growth, and various proteins associated with these processes were identified in tomato pollen, including pectin methylesterase (PME), pectin methylesterase inhibitor (PMEI), UTP-glucose-1-phosphate uridylyltransferase, UDP-glucose pyrophosphorylase, UDP-glucose:protein transglucosylase-like, microtubule-associated dTDP-glucose 4–6-dehydratase, and reversibly glycosylated protein. The PME enzyme catalyses the demethylesterification of homogalacturonans and plays an important role in pollen tube growth (Bosch *et al*., 2005; Chen and Ye, 2007). The post-translational modulation of PME activity is regulated by the enzyme PMEI, and both these enzymes were also reported in rice and *Arabidopsis* pollen (Table 1). Three GRPs were identified in tomato pollen, and the cell wall GRPs are suggested to have a structural function, probably acting as a scaffold or agglutinating agent for the deposition of cell wall constituents (Mousavi and Hotta, 2005). The presence of GRPs in tomato pollen could reflect their requirement during germination and tube growth.

Phragmoplastin, which is known to function in cell division and cell plate formation (Hong *et al*., 2003), was also identified in tomato pollen. The presence of phragmoplastin in mature tomato pollen could be related to its role in generative cell division, i.e. sperm cell formation, during pollen germination. Phragmoplastin was not reported in tri-cellular *Arabidopsis* and rice pollen.

Ca^{2+} binding and signalling

Ca^{2+} binding and signalling proteins such as annexin, calreticulin, and calmodulin were identified in tomato pollen, as in rice and *Arabidopsis* pollen (Table 1; Noir *et al*., 2005; Dai *et al*., 2006; Sheoran *et al*., 2006). Calcium and Ca^{2+}-binding proteins play important roles in pollen germination and tube growth (Taylor and Hepler, 1997; Golovkin and Reddy, 2003; Rato *et al*., 2004), and the presence of such proteins in mature pollen is indicative of the ready availability of Ca^{2+} required for these processes.

Hormone metabolism and signalling

The proteins aminocyclopropane-1-carboxylate (ACC) oxidase, IAA5 transcription factor, auxin-responsive calmodulin binding, and α-G protein (involved in hormone metabolism and signalling) were identified in tomato pollen (Table 1). ACC oxidase is one of the key enzymes in ethylene biosynthesis, and IAA5 transcription factor and auxin-responsive calmodulin-binding proteins play a significant role in auxin signalling. Both ethylene and auxin are known to regulate cell elongation, and these proteins could be required for pollen tube growth. Ethylene and auxin, or their precursors, have been reported in mature pollen (Singh and Sawhney, 1992; Holden *et al*., 2003). G proteins, which are heterotrimeric with α, β, and γ subunits, have a role in several plant developmental processes (Perfus-Barbeoch *et al*., 2005; Pandey *et al*., 2006) including modulation of cell division (Ullah *et al*., 2001; Chen *et al*., 2006). The presence of GPA1 in tomato pollen could be another factor involved in generative cell division. G proteins were not identified in *Arabidopsis* and rice pollen.

Pollen allergens

Pollen grains have been studied for allergen proteins in a number of species because of their allergenic activity toward humans (Mohapatra *et al*., 2005; Radauer and Breiteneder, 2006). A number of proteins in tomato pollen, such as profilins, luminal-binding proteins, and some of the Ca^{2+}-binding proteins discussed earlier, are also known to act as allergens. Allergenic proteins were also reported in rice and *Arabidopsis* pollen (Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Dai *et al*., 2006; Sheoran *et al*., 2006).

Other proteins

A number of proteins associated with nucleic acid, amino acid, lipid, and various other metabolic processes were identified. For example, six spots representing porins, which are localized in the outer mitochondrial membrane in various organisms and play a crucial role in the transport of metabolites between mitochondria and cytoplasm (Benz, 1994), were identified (Table 1). Seven spots were identified as hypothetical/unknown proteins with no well-defined function, as in other pollen proteomic studies (Holmes-Davis *et al*., 2005; Noir *et al*., 2005; Dai *et al*., 2006; Sheoran *et al*., 2006). An anther-specific protein, LAT52, with an established role in pollen development (Muschietti *et al*., 1994; McCormick, 2004), was also identified in tomato pollen.

Conclusions

This study has shown that tomato pollen contain various proteins with designated roles in defence mechanisms, energy metabolism, protein synthesis and processing, cytoskeleton formation, Ca^{2+} binding and signalling, and hormone signalling. The presence of these proteins in mature pollen is reflective both of the survival strategies of this small, two-celled independent structure and of the requirement for, and participation in, subsequent pollen germination and tube growth. Although no proteins specific for sperm cell formation were identified in mature tomato pollen, phragmoplastin and the α-G-protein may have a role in this process. Several new proteins not reported in the pollen of other species were identified in tomato pollen, including Pto-like serine/threonine kinase, coat protein (ToMV), HSP68 heat stress DnaK homologue, proteasome subunit-4, cystatin, IAA5 transcription factor, phragmoplastin, and α-G protein. Hence, this study (along with others) represents a significant contribution towards the construction of a comprehensive pollen proteome database encompassing many different species, which could serve as a valuable resource for researchers in plant biology in general, and in sexual plant reproduction in particular.

Acknowledgements

This research was supported by a Discovery grant from the Natural Sciences and Engineering Research Council of Canada to VKS, and by funding for mass spectrometry and proteomics equipment from the National Research Council of Canada.

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