

Dynamics of protein expression during pollen germination in canola (*Brassica napus*)

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Abstract The proteome of mature (MP) and in vitro germinating pollen (GP) of canola (*Brassica napus*) were analyzed using the DIGE technology with the objective of identifying proteins and their function in pollen germination. Of the 2,238 protein spots detected in gel images, 344 were differentially expressed in MP and GP samples of which 165 were subjected to MALDI-TOF/TOF and 130 were successfully identified using the NCBI and *Brassica* EST databases. The major proteins up-regulated in GP, relative to MP, have roles in carbohydrate metabolism, protein metabolism, and cell wall remodeling. Others with roles in cytoskeleton dynamics, nucleotide and amino acid metabolism, signal transduction, and stress response also showed higher expression in GP. Proteins concerned with transcriptional regulation and ion transport were similar in MP and GP, and some catalases and LEA proteins were down-regulated in GP. A number of proteins including, oleosin, cruciferin, and enolase, were released into the pollen germination medium indicating their potential role in pollen–stigma interaction. Glycosylated proteins were also identified in MP and GP, but their protein profiles were not different. This study has documented the dynamics of protein expression during pollen germination and early tube growth in *B. napus* and provides insights into the

fundamental mechanisms involved in these processes, and in cell growth, cell–cell communication, and cell signaling.

Keywords *Brassica* · Canola · Differential in-gel electrophoresis (DIGE) · Mass spectrometry · Pollen · Pollen germination · Proteomics

Abbreviations

2-DE	2-Dimensional gel electrophoresis
DIGE	Differential in-gel electrophoresis
MP	Mature pollen
GP	Germinating pollen
PGM	Pollen germination medium

Introduction

In seed plants, pollen grains, or the male gametophytes, serve as the vehicles to transport sperm cells to the female gametophyte for fertilization. The entire process beginning with pollen formation and ending in sperm release involves several steps; the development of pollen grains, their dispersal and transfer to the female reproductive organ by agents, e.g., wind and insects, pollen germination, pollen tube growth and its penetration of the female gametophyte, and the release of sperm cells. Each of these steps has been examined in detail in a number of plant species (see, e.g., Mascarenhas 1990, 1993; Shivanna 2003; Scott et al. 2004; Weterings and Russell 2004; Malhó 2006). At the genetic level, genes expressed during pollen development (e.g., Honys and Twell 2003; McCormick 2004), the presence of pollen-specific transcripts in mature pollen (Becker et al. 2003; Honys and Twell 2003; Pina et al. 2005), and gene expression during pollen germination and tube growth (Mascarenhas 1993; Hepler et al. 2001; Wang et al. 2008)

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have received special attention. A large number of transcripts related to cell wall metabolism, cytoskeletal dynamics, and cell signaling have been identified in mature pollen, and significant changes in transcripts related to calmodulin/calmodulin-like proteins, cation/hydrogen exchanger, heat shock proteins, and signal transduction, were reported during pollen germination.

With major improvements in proteomic technologies, including protein separation and high-throughput mass spectrometry techniques, and the availability of genomic databases, emphasis has also been placed on understanding the roles of proteins, the functional molecules of cells and tissues, in various aspects of pollen development, maturation, germination, and tube growth. Proteomic changes during anther development (Kerim et al. 2003; Miki-Hirosige et al. 2004) and the proteome of mature pollen (Holmes-Davis et al. 2005; Noir et al. 2005; Dai et al. 2006; Sheoran et al. 2006, 2007; Li et al. 2008) have been examined in a few species. It has been shown that the mature pollen grains contain a number of proteins associated mainly with carbohydrate/energy metabolism, protein metabolism, cytoskeletal formation, Ca^{2+} signaling, wall modeling, hormonal signaling, and defense mechanisms (see review Dai et al. 2007b; Sheoran et al. 2007). In the species examined, proteins with roles in energy metabolism were the major component of mature pollen, unlike their corresponding transcripts, indicating that the pollen grains contain pre-synthesized proteins required for germination and tube growth, supporting an earlier held view (Mascarenhas 1993; Shivanna 2003).

Pollen germination is essential for the reproductive success of angiosperms and gymnosperms and several physiological and biochemical mechanisms involved in pollen germination, and rapid tube growth have been investigated (see, e.g., Franklin-Tong 1999; Shivanna 2003; Malhó 2006). A number of proteins with roles in pollen hydration, pollen–stigma interaction, germination and tube growth, including kinases, proteasomes, profilin, pectin methylesterase (PME), polygalacturonase (PG), and calcium-binding proteins, were identified during pollen germination and tube growth (Hepler et al. 2001; Sheng et al. 2006; Hiscock and Allen 2008), and some glycosylated proteins were shown to be specifically synthesized during pollen tube growth (Čapková et al. 1994). Recently, proteomic analyses of mature and germinating pollen of rice (Dai et al. 2007a) and two gymnosperm species, *Pinus strobus* (Fernando 2005) and *Picea meyeri* (Chen et al. 2006), were conducted and proteins associated with carbohydrate/energy metabolism, protein metabolism, and wall modeling were up-regulated during germination in these species. However, these studies used CBB staining for detection of proteins, as opposed to more sensitive methods such as differential in-gel electrophoresis (DIGE) which uses

fluorescent dyes (Marouga et al. 2005), and, therefore, it limited the detection of many differentially expressed proteins. Also, the pollen proteins secreted during germination were not analyzed.

Brassica napus (canola) is one of the major oilseed crops in the world, produces a large amount of pollen, and is a major source of honey production. Pollen development, including the analyses of male-sterile mutants (Polowick and Sawhney 1990; González-Melendi et al. 2008), factors affecting pollen germination and tube growth (Shivanna and Sawhney 1995), and the molecular mechanisms involved in self-incompatibility (Watanabe et al. 2001), have been investigated in some *Brassica* species. A number of genes expressed in *B. napus* pollen correlate with known pollen proteins, such as pectin esterase, polygalacturonase, pectin esterase inhibitor, and arabinogalactan proteins (Robert et al. 1993; Treacy et al. 1997). Some of the pollen coat proteins, e.g., oleosin-like proteins, protein kinase, and pectin esterase were also identified in *Brassica* pollen (Murphy 2006). However, to our knowledge, the proteome of mature *B. napus* pollen and in particular, the changes in protein expression during pollen germination have not been investigated. This information is fundamental to our understanding of the mechanisms involved in the survival of the small, free-floating, pollen grains, and their function in the delivery of sperm cells for fertilization.

Thus, the goal of this study was to analyze the proteome of *B. napus* mature pollen and examine the dynamics of protein expression during pollen germination, with the objective of identifying key proteins in mature and germinating canola pollen, and determining their possible roles during germination and early tube growth. The mature and in vitro germinated pollen were analyzed by the DIGE technology and a total of 2,238 protein spots were observed in 2-DE gels, of which 1,554 were consistently present in replicates, and 344 were differentially expressed (with twofold change or greater) between mature and germinating pollen. In addition, a number of proteins released into the germination medium were identified. The data presented provide insights into the proteome of mature canola pollen and their functional roles during germination and early tube growth.

Materials and methods

Plant growth and pollen collection

Canola (*B. napus* cv. Westar, seeds provided by Agriculture and Agri-Food Canada, Saskatoon Research Centre, Saskatoon) plants were grown in 16-cm plastic pots containing Tera-lite Redi-earth mix and placed in a growth chamber under 26/23°C (day/night), and 16/8 h light/dark conditions. Light was supplied by fluorescent tubes (F72T12/

CW/VHO; Sylvania, Danvers, MA, USA) and incandescent bulbs at a photon flux density of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were fertilized once a week using 20-20-20 (Plant Products Co. Ltd, Ajax, ON, Canada). Mature pollen (MP) were collected from freshly opened flowers by tapping anthers on a glass slide, checked under a dissecting microscope, and any debris removed with a fine needle. Pollen samples were pooled in an Eppendorf tube and the purity of samples again determined under a light microscope. Pollen grains were either used immediately or stored at -80°C for later use. Approximately 100 mg of pollen was collected from ~1,000 flowers (35 plants) for each biological replicate, and four biological replicates used for each analysis.

Pollen germination

Pollen grains were initially incubated for 2 h at 100% humidity and then cultured in Roberts's liquid growth medium (Roberts et al. 1983) composed of 20% w/v sucrose, 3.0 mM CaCl_2 , 1.0 mM KNO_3 , 0.16 mM H_3BO_3 and 0.66 mM Tris, and adjusted to pH 8.0 in a glass centrifuge tube ($100 \mu\text{l mg}^{-1}$ pollen). The tubes were placed on a rotary shaker, slanted at an angle of approximately 35° , and the pollen grains allowed to germinate for 1 h at room temperature with gentle shaking (80 rpm). At various time intervals during 1 h, 5- μl samples were withdrawn and germination was recorded under a light microscope. The germinating pollen (GP) were collected by centrifugation at $500\times g$ for 5 min at 4°C and used immediately for protein extraction.

Protein extraction from mature (MP) and germinating pollen (GP)

Mature (~50 mg) and germinating pollen (~25 mg, after 1 h of incubation), were ground separately to a fine powder in a pestle and mortar in the presence of liquid nitrogen and extracted with TCA–acetone (Sheoran et al. 2007). After four washes with acetone, the residue was vacuum dried and proteins extracted in a urea buffer comprising 8 M urea and 4% CHAPS by extensive vortexing for 1 h at room temperature. The solution was centrifuged at $20,000\times g$ for 20 min at 20°C and the supernatant collected. The resulting pellet was re-extracted with urea buffer and the supernatants combined. The protein amount was then estimated using the Bio-Rad DC protein Assay Kit (Bio-Rad, Hercules, CA, USA) and samples were either used immediately or stored at -80°C for later use.

Protein extraction from pollen germination medium (PGM)

The supernatant pollen germination medium (PGM), collected after removal of germinating pollen, was centrifuged

at $2,000\times g$ for 15 min at 4°C to remove any debris. Four volumes of 100% acetone were then added, and proteins allowed to precipitate at -20°C for 1 h before centrifuging at $20,000\times g$ for 15 min at 4°C . The pellet was washed four times with 80% acetone and once with 100% acetone, vacuum dried, and proteins extracted with the urea buffer.

Protein-cyanine dye labeling

The proteins extracted from MP, GP, and PGM were labeled for DIGE using fluorescent cyanine dyes according to the manufacturer's instructions (GE Healthcare, Baie d'Urfé, Canada), as described previously (Sheoran et al. 2009). All procedures from dye labeling to scanning of gels were performed in the dark or under diffused light. Each protein sample (50 μg) was labeled with 250 pmol of Cy2, Cy3 or Cy5 amine-reactive cyanine dyes, following pH adjustment. Each protein sample was mixed with one of the dye solutions, vortexed, centrifuged briefly, and incubated on ice for 30 min in the dark. The reaction was terminated by adding 1 μl of 10 mM lysine, which quenches the remaining free dye, and incubated on ice for 10 min in the dark. An equal volume of urea buffer (8 M urea, 120 mM DTT, 4% CHAPS, and 2% ampholyte, pH 4–7 or 3–11) was added to each sample, mixed and kept on ice for 15 min in the dark.

Four biological replicates each of the MP and GP extracts, and two of the PGM extracts, were labeled with cyanine dyes. The replicate samples were labeled alternatively either with Cy3 or Cy5. A 'pooled internal standard' sample was prepared by mixing equal quantities of protein from all samples and labeling with Cy2 to allow for gel normalization between replicates. The Cy2-labeled samples were then combined with Cy3 and Cy5 labeled samples for 2-DE separation.

Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis was carried out according to the established procedures (Sheoran et al. 2007). IEF was performed using the Multiphor II horizontal electrophoresis system (Amersham Biosciences, Uppsala, Sweden) and 18 cm Immobiline Dry Strips of 4–7 or 3–11 non-linear pH gradients. The 4–7 pH strips were rehydrated overnight in a solution containing 8 M urea, 2% CHAPS, 20 mM DTT, 0.002% bromophenol blue, 2% IPG buffer (pH 4–7), and Cy2, Cy3, and Cy5 labeled mixed protein samples. An additional 200 μg of unlabeled protein from MP and GP samples was also included to enhance spot detection with colloidal coomassie blue (CCB) G-250, as required for spot picking and MS analysis following fluorescence detection. The pH 3–11 strips were rehydrated in Destreak-Rehydration solution (GE Healthcare)

overnight without the protein samples. The protein samples were then cup-loaded on these strips as per the standard procedure. 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 100 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 in place of DTT. The equilibrated strips were applied to vertical SDS-polyacrylamide gels (12.5%), cast in low fluorescent glass plates, and sealed with 0.5% agarose in SDS buffer containing bromophenol blue. Electrophoresis was performed for 30 min at 15 and then at 20 mA gel⁻¹ until the dye front reached the bottom of the gel. Six gels were run simultaneously in a PROTEAN II XI multi-cell (Bio-Rad).

Gel imaging and analysis

Following electrophoresis, the gel plates were washed with distilled water, wiped dry, and scanned using the Typhoon Variable Mode Imager 9400 (GE Healthcare, Buckinghamshire, UK) set at 100 µm resolution. Images of the Cy2-, Cy3-, and Cy5-labeled proteins in each gel were acquired by excitation at 488, 532, and 633 nm and emission at 520, 590, and 680 nm, respectively. After scanning, the gels were stained with CCB for spot picking. The scanned gel images were processed using Image Quant V 5.2 software (GE Healthcare) for spot area and color contrast. Spot normalization, spot volume ratio, and statistical analysis (Student's *t*-test and one-way ANOVA) of differentially expressed spots were performed by using DeCyder V 6.05.11 software (GE Healthcare).

Protein identification by mass spectrometry

Based on image analysis results, a total of 165 spots, 34 spots showing a twofold or higher increase, and 103 with twofold or greater decrease in spot intensity (expressed as spot volume) in MP over GP, and 28 highly abundant spots but with less than twofold change, were selected from CCB-stained gels for MS analysis (spots were matched by overlaying CCB-stained and DIGE images). 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 C18 ZipTips (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's instructions. The digests were then analyzed using 4800 MALDI-TOF/TOF Analyzer (Applied Biosys-

tems, Framingham, MA, USA) as described previously (Sheoran et al. 2009).

Plate modeling and default calibration of the MALDI-TOF/TOF instrument were carried out using a peptide mixture consisting of Angiotensin 1 (*m/z* 1296.6853), ACTH 1–17 (*m/z* 2093.0867), ACTH 18–39 (*m/z* 2465.1989), and ACTH 7–38 (*m/z* 3657.9294). The mixture was applied to the MALDI target plate at pre-defined calibration positions, mixed (1:1) on-plate with CHCA matrix (5 mg ml⁻¹ in 75% acetonitrile containing 0.1% TFA and 10 mM ammonium phosphate), and dried under a gentle stream of warm air. Digest samples were applied to the plate in a similar fashion. Calibration for MS/MS mode was carried out using fragment ion masses from Glu-fibrinogen. Initial analysis of protein digests on the 4800 instrument was carried out by MS in positive ion reflectron mode, using trypsin autolysis products (*m/z* 842.510, 1045.564 and/or 2211.105) for internal mass calibration where possible, or else the default calibration. MS spectra were generated by combining 800 laser shots per sample. The ten most abundant precursor ions in each spectrum (excluding trypsin autolysis fragments) were subsequently selected by GPS Explorer 3.5 software for MS/MS analysis, which was performed using air as the collision (CID) gas, increased laser fluence, and 2000 laser shots collected per sample.

Processed spectra from the above experiments were submitted by GPS Explorer 3.5 software to MASCOT for combined (MS + MS/MS) database searches, using carbamidomethylation of cysteine as the fixed modification and oxidized methionine as a variable modification, allowing 100 ppm and 0.6 Da errors for MS (peptide ion) and MS/MS (fragment ion) data, respectively. The NCBI nr (viridiplantae date: 24/07/08) and *Brassica* species EST (date 24/07/08, 582504 sequences) databases were searched using Mascot search engine (Matrix Science).

Glycoprotein staining

The MP and GP protein samples were separated by 1- and 2-DE and stained with Krypton™ Glycoprotein Stain (Pierce, Rockford, USA). For 1-DE, 25 µg of protein from each of MP and GP samples was separated on 12.5% SDS-polyacrylamide gels, whereas 2-DE analysis was carried out as described above. The gels were stained with glycoprotein-specific stain as per the manufacturer's instructions, and subsequently with CCB for visualization of all proteins.

Results

Figure 1 shows the mature and germinating canola pollen after 1 h of in vitro culture. Germination was initiated

Fig. 1 Mature (a) and in vitro germinating pollen after 1 h of incubation (b), of canola (*B. napus*)

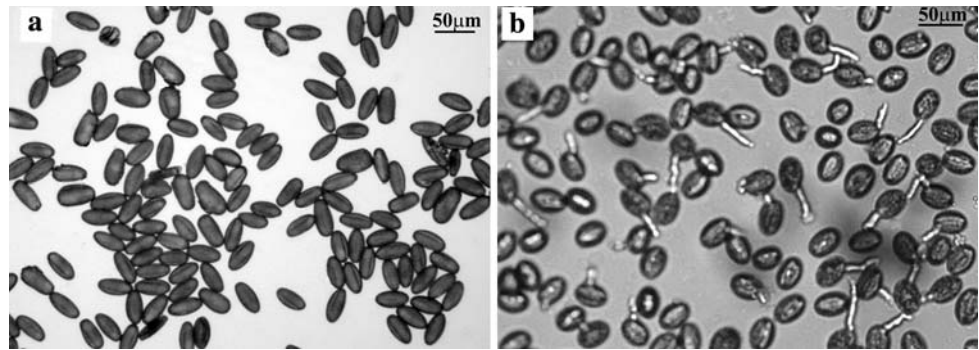
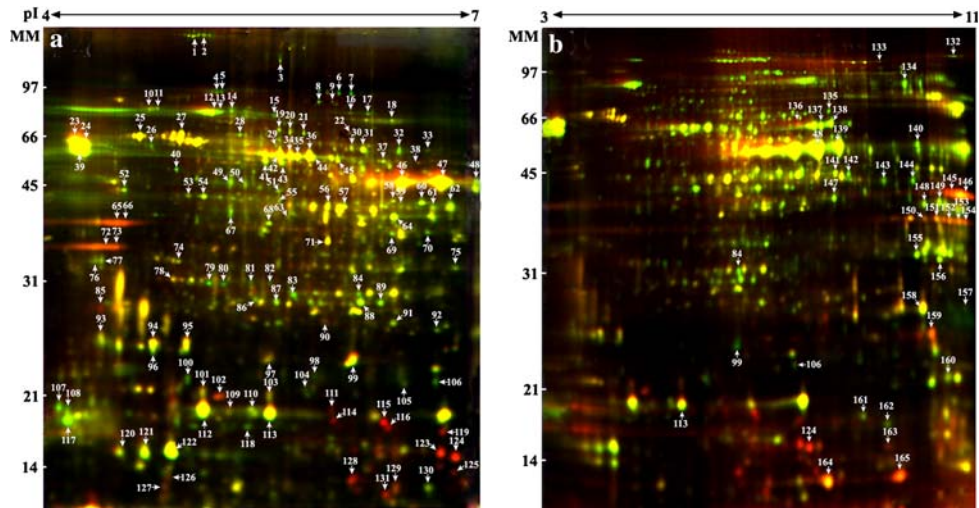


Fig. 2 DIGE gel images of mature (MP) and 1 h germinating pollen (GP) of *B. napus* labeled with cy5 (red) and cy3 (green), and proteins separated with 4–7 pH IPG strips (a) and 3–11 pH IPG strips (b). Red spots indicate highly expressed proteins in MP and green spots in GP, and the yellow spots represent similar level of expression in both. Molecular markers (in kDa) are shown on the left and the numbered spots were used for mass spectrometry analysis



within 15 min of culture, and maximum germination was observed after 1 h (mean $74.8 \pm 5.3\%$). Mature pollen were oblong in shape, and changed from 45 to 35 μm in length, and from 20 to 25 μm in width, during germination.

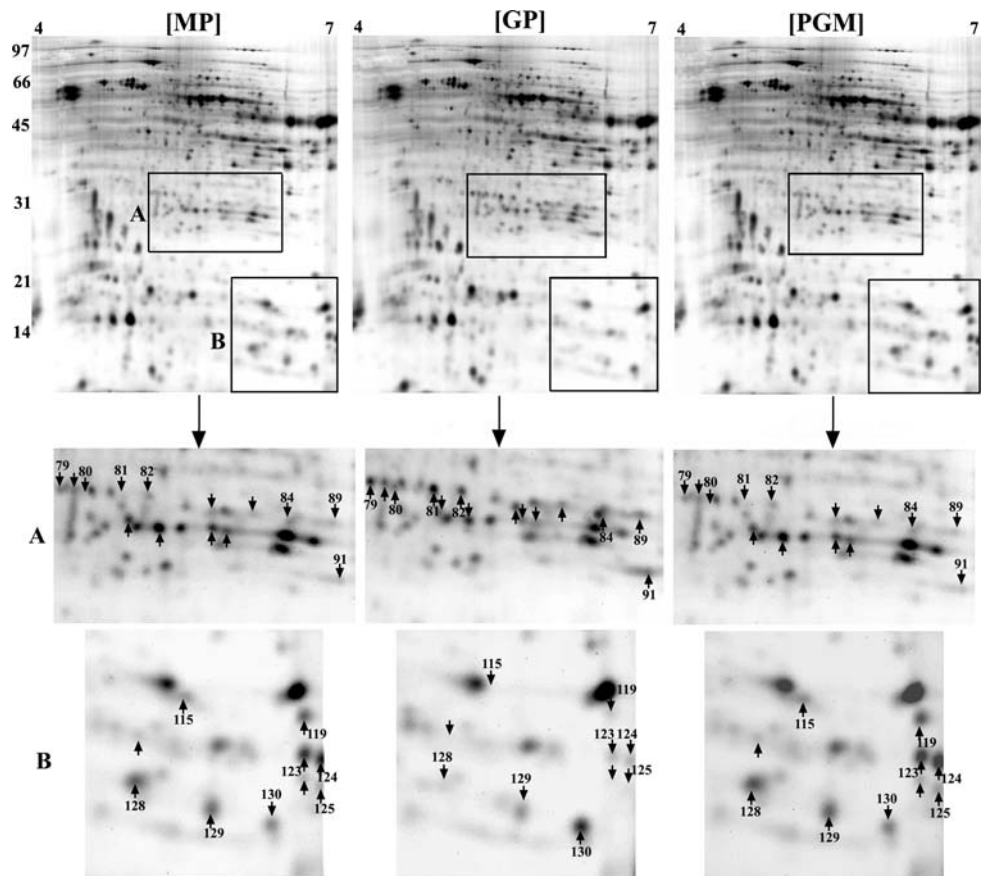
Differential expression of proteins in MP and GP samples was analyzed by DIGE using both pH 4–7 and 3–11 IPG strips, and the representative images are presented in Fig. 2. Red spots indicate higher expression in MP and green spots in GP, relative to the other sample. Yellow spots indicate a similar level of expression in both MP and GP samples. In the interest of clarity, the DIGE gel images presented in Fig. 2 do not include the Cy2-labeled internal standard proteins.

Spot analysis of pH 4–7 DIGE image using DeCyder software revealed a total of 2,238 spots, of which 1,554 matched consistently in all four replicates. Analysis of the pH 3–11 gels detected 2,052 spots and of these, 1,400 matched across all replicates. Biological Variance Analysis (BVA) of pH 4–7 gel images using the DeCyder software revealed 88 and 112 spots with a twofold or higher ($P \leq 0.05$, $n = 4$) level of expression in MP and GP, respectively (total 210 spots). For pH 3–11 gels a total of 344 spots were differentially expressed (183 for MP and 161 for GP samples).

The overall protein profile on gel images (pH 4–7) of MP, GP, and PGM appeared similar (Fig. 3), although there were differences in the level of expression in each sample, i.e., some proteins were up- and others down-regulated (see enlarged portions of gels in Fig. 3). The high number of protein spots in the PGM sample may, in part, be due to the bursting of some pollen grains/tubes and resulted in the release of some proteins into the medium. Nevertheless, it should be noted that some spots (marked by arrows) were of higher expression in MP relative to GP, while others which were of lower expression in GP, relative to MP, were of higher intensity in PGM (Fig. 3).

Based on DIGE gel image analysis, a total of 165 spots were subjected to MALDI-TOF/TOF mass spectrometry for protein identification. Of these, 130 (78%) were analyzed successfully, resulting in the identification of 80 distinct proteins using NCBIInr (viridiplantae) and *Brassica* EST databases (Table 1). Proteins with significant MAS-COT protein and ion score, with at least four peptide mass and two peptide sequence matches, with a minimum of 10% sequence coverage, were considered as identified. Searches against the NCBIInr protein database were not very successful, due to under-representation of *Brassica* protein sequences in the database. However, the availability

Fig. 3 2-DE gels of mature (MP), germinating (GP) and the pollen germination medium (PGM) and stained with CCB. The boxes A and B in the three gels were enlarged (below) and the numbered spots with arrows up and down represent proteins up- and down-regulated, respectively. Molecular markers (in kDa) are shown on the left



of *B. napus* ESTs (NCBI) allowed large numbers of *Brassica* proteins to be identified with high confidence. Although unidentified, the remaining 35 spots produced good-quality MS data, suggesting that they may contain proteins (possibly unique to pollen) whose sequences are not available in the database, or which may be extensively modified.

The differentially expressed proteins in MP and GP samples were grouped into 13 functional categories (Fig. 4a) based on their known protein functions. The largest single group of identified proteins belonged to carbohydrate and energy metabolism category (29%), followed by protein metabolism (21%), and wall remodeling (15%). These three groups together constituted 65% of the total proteins identified. The other groups were signal transduction (6%, mostly proteins involved in Ca^{2+} -binding and signaling), stress response (6%), nucleotide metabolism (4%), amino acid metabolism (3%), cytoskeleton dynamics (3%), ion transport (3%), and miscellaneous (4%).

The number of protein spots in each functional group with a twofold or greater increase in spot intensity in MP and GP samples is presented in Fig. 4b. Within the carbohydrate and energy metabolism group, 3 protein spots were more intense in MP but 29 in GP samples, whereas for the protein metabolism group the numbers were 3 and 21, and

for wall remodeling, 2 and 14, respectively. Differentially expressed proteins with roles in amino acid and nucleotide metabolism, together with the unknown proteins, were identified only in GP samples, whereas for the remaining functional groups a similar number of proteins were identified in GP and MP samples (Fig. 4b). Hence, more proteins were up-regulated in GP than in MP samples.

The presence of glycosylated proteins in the reproductive organs of many plant species, and their suggested role in pollen germination, tube growth, and self-incompatibility, prompted us to analyze glycosylated proteins in MP and GP samples. Many of the proteins in MP and GP samples were glycosylated (Fig. 5) although the expression profiles of such proteins were similar. In 1-DE gels, approximately 15 glycoprotein bands (Fig. 5a) and in 2-DE gels 42 glycosylated protein spots (Fig. 5b) were identified for both MP and GP samples.

Discussion

Mature pollen grains store a number of substances, including polysaccharides, proteins, lipids, and hormones, which play important roles in pollen germination and early tube growth. These developmental events, which involve cell

Table 1 Proteins identified in the mature and germinating pollen of *B. napus* using MALDI-TOF/TOF mass spectrometry

Spot no.	Gene ID	Species	Protein identity	MM/pI ^a	Protein score ^b	Ion score ^c	Spot volume ratio (GP/MP) ^d	p-Value
Carbohydrate and energy metabolism								
6	23297457	AT	Putative 2,3-biphosphoglycerate-independent phosphoglycerate mutase	60.6/5.32	423	348	2.57	0.0080
7	23297457	AT	Phosphoglycerate mutase	60.6/5.32	512	413	2.77	0.0019
8	4586021	AT	Cytoplasmic aconitate hydratase	98.1/5.79	520	439	2.78	0.0050
16	30693102	AT	EMB1467 (embryo defective 1467); NADH dehydrogenase	81.1/6.24	283	172	2.02	0.0073
17	30693102	AT	NADH dehydrogenase	81.1/6.24	156	86	2.42	0.013
19	18391066	AT	Phosphoglycerate mutase	60.5/5.32	627	444	2.39	0.0050
20	18391066	AT	Phosphoglycerate mutase	60.5/5.32	875	645	2.04	0.0025
21	21537260	AT	Putative phosphoglycerate mutase	60.9/5.50	752	518	2.28	0.0072
32	140325934	BJ	ATPase alpha subunit	55.0/6.01	170	96	3.08	0.0047
34	186521400	AT	ATP synthase beta chain 1, mitochondrial	59.6/6.13	354	224	2.01	0.0260
35	186521400	AT	ATP synthase beta chain 1, mitochondrial	59.6/6.13	663	449	2.31	0.0012
36	34597332	BN	Enolase	47.3/5.78	487	294	1.73	0.0100
42	13548325	AT	H ⁺ -transporting ATP synthase beta chain	59.8/6.2	352	214	1.64	0.0099
44	34597332	BN	Enolase	47.3/5.78	387	194	2.03	0.0026
50	23397307	AT	Unknown protein similar to ATPase	48.3/5.41	250	162	2.56	0.0003
51	23397307	AT	Unknown protein similar to ATPase	48.3/5.41	178	132	2.21	0.0025
64	15219721	AT	Putative cytosolic malate dehydrogenase (MDH)	35.9/6.11	95	82	2.00	0.0340
66	34597330	BR	Enolase	47.6/5.5	232	141	−2.85	0.0064
68	15224669	AT	pfkB-type carbohydrate kinase family protein	35.3/5.31	231	185	2.18	0.0022
69	21593602	AT	Putative MDH	35.9/6.1	261	195	2.09	0.0610
71	70611326	BN	Glyceraldehyde 3-P dehydrogenase	17.6/5.4	254	131	1.16	0.0670
74	15227987	AT	LOS2 (low expression of osmotically responsive genes 1); phosphopyruvate hydratase, enolase	47.7/5.5	103	99	1.14	0.0061
78	20197320	AT	Putative inorganic pyrophosphatase	24.9/5.55	252	184	−1.15	0.0110
79	26451240	AT	Putative inorganic pyrophosphatase	24.9/5.70	145	122	6.46	0.0025
80	26451240	AT	Putative inorganic pyrophosphatase	24.9/5.70	198	137	8.77	0.0003
83	22326673	AT	ATP synthase beta chain, mitochondrial, putative	58.8/6.0	382	321	2.75	0.0008
87	15233272	AT	Cytosolic triose-phosphate isomerase	27.1/5.39	369	278	2.16	0.0025
88	13432260	AT	Cytosolic triose-phosphate isomerase	27.1/5.39	483	406	2.44	0.0025
96	17939851	AT	F0 ATP synthase D chain	17.2/5.0	384	205	2.04	0.0013
105	70611326	BN	Glyceraldehyde-3-phosphate dehydrogenase	17.6/5.4	276	178	2.16	0.0170
125	15235431	AT	Hydrogen ion transporting ATP synthase	14.2/6.1	182	114	−3.58	0.0067
128	15235431	AT	Hydrogen ion transporting ATP synthase	14.2/6.1	253	203	−7.57	0.0013
144	30680156	AT	Fructose-bisphosphate aldolase, putative	38.6/7.6	225	187	5.08	0.0100
148	15227752	AT	PMDH1(Proxisomal malate dehydrogenase 1)	37.4/8.11	337	294	2.07	0.0900
149	15227752	AT	PMDH1(Proxisomal malate dehydrogenase 1)	37.4/8.11	105	95	2.04	0.0040
155	15238025	AT	NADH-cytochrome b5 reductase putative	31.5/8.57	118	99	2.21	0.0061
Protein metabolism								
4	18414193	AT	Cell division control protein 48 homolog E	89.9/5.08	486	285	2.63	0.0084
5	98962497	NT	Putative spindle disassembly related protein CDC48	89.9/5.13	173	119	2.80	0.0041
9	29824421	AT	Putative elongation factor	74.0/6.91	322	162	2.43	0.0025
10	397482	AT	Heat shock protein 70 cognate	71.6/5.0	209	104	1.81	0.0065
11	18175969	AT	Putative HSP70 protein	76.6/5.1	117	90	2.11	0.0069

Table 1 continued

Spot no.	Gene ID	Species	Protein identity	MM/pI ^a	Protein score ^b	Ion score ^c	Spot volume ratio (GP/MP) ^d	p-Value
12	30693966	AT	BIP (luminal binding protein); ATP binding	67.4/5.18	312	164	3.08	0.0084
13	30693962	AT	BIP (luminal binding protein); ATP binding	67.4/5.18	424	243	3.56	0.0051
14	37718900	OS	Putative heat shock protein partial	82.5/5.43	131	118	3.85	0.0025
15	30794015	AT	Dipeptidyl peptidase IV	84.3/6.3	225	176	2.40	0.0020
22	6513923	AT	Putative mitochondrial processing peptide	59.2/6.3	126	108	2.22	0.0130
26	77999357	BC	Protein disulfide isomerase	55.7/5.0	238	198	2.36	0.0043
28	15229559	AT	HSP60; ATP binding; protein binding	61.2/5.66	228	171	2.43	0.0048
31	15232845	AT	MPPBETA; metalloendopeptidase	59.1/6.3	273	106	2.53	0.0054
40	15230005	AT	RPT5A (regulatory particle triple-A 5A) ATPase/calmodulin binding	47.4/4.9	197	183	2.75	0.0160
53	18405292	AT	Ubiquitin-protein ligase	45.0/5.4	133	96	2.05	0.0050
77	30687350	AT	Elongation factor 1B alpha subunit 2	24.2/4.4	210	177	2.21	0.0025
89	15224993	AT	20S Proteasome subunit PAA2	27.4/5.75	181	99	2.25	0.0031
91	79318062	AT	20S Proteasome α -subunit B	25.7/5.5	240	128	2.09	0.0028
93	19805	NT	Luminal binding protein	32.1/4.6	128	82	2.00	0.0160
102	23396587	AT	Peptidyl-prolyl <i>cis-trans</i> isomerase	17.6/5.27	204	181	−2.87	0.0002
118	40805177	BN	Eukaryotic translation initiation factor-5A	17.0/5.7	196	108	2.16	0.0056
131	33309455	BR	Phytocystatin	11.3/5.5	106	97	−4.40	0.0040
135	15222666	AT	Chaperonin, putative	66.8/9.22	215	189	3.89	0.0064
154	12751303	BN	Prohibitin 1-like protein	32.0/9.45	117	95	−2.42	0.0033
160	15236211	AT	Peptidyl-prolyl <i>cis-trans</i> isomerase	18.3/8.9	108	99	2.03	0.0037
Wall remodeling and metabolism								
1	30685162	AT	Leucine-rich repeat family protein/ extensin family protein	102.8/5.46	233	175	2.00	0.0068
2	30685162	AT	Leucine-rich repeat family protein	102.8/5.46	112	97	2.09	0.010
41	30686293	AT	UDP-glucose pyrophosphorylase	43.7/7.6	474	378	2.06	0.0047
43	30686293	AT	UDP-glucose pyrophosphorylase	43.7/7.6	552	487	2.08	0.0003
45	15241704	AT	UDP-glucose 6-dehydrogenase, putative	53.0/5.6	321	242	2.69	0.0035
46	21668071	BO	Putative pollen polygalacturonase	42.3/6.6	306	234	1.60	0.0039
47	30984029	BN	Polygalacturonase	42.3/6.6	400	332	1.64	0.0065
48	548490	BN	Polygalacturonase precursor (PG) (Pectinase)	42.4/6.3	578	504	1.99	0.0069
55	2317729	AT	Reversibly glycosylated polypeptide-1	41.2/5.6	247	158	2.00	0.0065
63	2317729	AT	Reversibly glycosylated polypeptide-1	40.7/5.6	329	199	2.34	0.0088
75	30984029	BN	Pollen polygalacturonase	43.2/6.6	238	147	2.30	0.0038
81	56480906	BR	Xyloglucan endotransglycosylase/hydrolase	31.9/5.17	133	85	2.92	0.0110
84	548490	AT	Polygalacturonase precursor (PG) (pectinase)	42.4/6.3	587	497	3.31	0.0023
94	168314	LP	Pollen allergen	27.7/5.4	125	81	1.05	0.0150
101	1857671	BN	Pollen-specific protein Bnm 1	19.8/5.3	237	179	1.68	0.0061
103	1857671	BN	Pollen-specific protein Bnm 1 Similar to pectinesterase inhibitor	19.8/5.3	198	109	1.66	0.0230
111	15233902	AT	Pectinesterase inhibitor	19.9/5.54	257	198	−2.51	0.0025
112	1857671	BN	Pollen-specific protein Bnm 1	19.8/5.3	358	256	−1.10	0.1900
113	1857671	BN	Pollen-specific protein Bnm 1	19.8/5.3	162	116	−1.17	0.1500
119	18423677	AT	Pollen ole e 1 allergen	16.1/6.88	178	115	−4.54	0.0041
132	4581116	AT	Putative beta-galactosidase	94.0/8.76	101	84	7.10	0.0084
133	1352075	BO	Beta-galactosidase precursor (lactase)	93.0/7.93	352	223	2.18	0.0180

Table 1 continued

Spot no.	Gene ID	Species	Protein identity	MM/pI ^a	Protein score ^b	Ion score ^c	Spot volume ratio (GP/MP) ^d	p-Value
134	42569272	AT	Glycoside hydrolase family 28 protein/polygalacturonase (pectinase)	51.6/8.78	457	416	3.47	0.0740
139	30984029	BN	Polygalacturonase	42.3/6.6	306	234	1.36	0.0060
Cytoskeleton dynamics								
49	166582	AT	Actin-1	41.7/5.3	428	348	2.43	0.0043
67	4139264	BN	Actin	41.6/5.3	237	153	2.13	0.0020
121	158122108	BN	Profilin	14.5/5.2	120	92	1.39	0.0140
122	11229030	BN	Profilin	14.6/5.0	238	170	1.43	0.0120
126	11229030	BN	Profilin	14.6/5.0	131	84	−2.76	0.0025
Signal transduction								
23	2429343	BN	Calreticulin	47.8/4.6	229	193	1.09	0.0210
24	21555174	AT	Calcium-binding protein, calreticulin	48.4/4.4	122	96	−1.23	0.0120
70	6503082	AT	AnnAt3 (annexin 3 calcium binding)	36.5/6.1	133	80	2.15	0.0110
107	30683369	AT	Calmodulin 5	20.6/4.7	190	104	2.09	0.0120
108	28192990	BO	Calmodulin 1	15.5/4.2	258	180	2.21	0.0058
117	15221593	AT	Calcium ion binding	16.6/4.9	239	192	4.07	0.0023
145	1550740	AT	GDP associated inhibitor	49.8/5.49	189	138	−3.72	0.0039
152	15233228	AT	Receptor-like protein kinase-related	30.3/9.15	175	99	−2.23	0.0088
Stress response								
33	4336756	BJ	Catalase	57.2/6.9	214	127	3.43	0.0120
38	4336754	BJ	Catalase	56.8/6.63	238	187	−2.23	0.0020
86	18265379	BO	Ascorbate peroxidase	27.7/5.58	151	95	2.44	0.0041
110	17819	BN	Glycine-rich RNA-binding protein	16.3/5.6	157	115	2.07	0.0046
136	169244543	BN	Catalase	57.1/6.75	120	97	−4.37	0.0012
137	2511725	AT	Catalase 1	57.1/7.14	162	127	3.59	0.0054
158	15233425	AT	L-Ascorbate peroxidase 5	30.9/8.80	246	187	−1.40	0.0032
164	15236313	AT	UNE15 (unfertilized embryo sac 15) Similar to LEA protein	11.6/6.71	146	126	−2.38	0.0160
Transcriptional regulation-related								
52	28393236	AT	SET-domain transcriptional regulator	45.0/4.7	230	119	2.06	0.0014
151	5903072	AT	Transcription factor	37.6/9.6	178	104	−2.31	0.0021
Amino acid metabolism								
29	7270370	AT	Phosphoglycerate dehydrogenase-like protein	63.3/6.16	272	210	2.02	0.0099
57	11386845	AT	Glutamine synthetase cytosolic	38.6/5.7	141	96	1.91	0.0028
60	15229530	AT	Glutamine synthase clone KB6	38.6/5.7	285	217	2.98	0.0021
147	15224592	AT	Aspartate aminotransferase 1	47.7/8.36	472	336	2.75	0.0120
Nucleotide metabolism								
18	15231167	AT	Unknown protein (similar to cell cycle protein)	78.7/6.8	124	78	1.99	0.0073
25	15229825	AT	Spliceosomal protein U1A/RNA-binding	50.3/9.4	99	78	1.20	0.0720
54	15242717	AT	ADK2 (adenosine kinase 2); kinase	37.8/5.1	576	472	2.69	0.0065
92	18415727	AT	Endonuclease/nucleic acid binding	33.9/6.3	136	95	2.04	0.0170
161	18413214	AT	ATP binding/nucleoside diphosphate kinase	18.8/8.4	91	79	2.34	0.0520
Lipid metabolism								
140	15225798	AT	KAT2/PED1 (peroxisome defective1); acetyl-CoA C-acyltransferase	48.6/8.62	291	227	4.80	0.0028
163	15234945	AT	OLEO1 (oleosin1)	18.5/9.43	151	114	−9.48	0.0042

Table 1 continued

Spot no.	Gene ID	Species	Protein identity	MM/pI ^a	Protein score ^b	Ion score ^c	Spot volume ratio (GP/MP) ^d	p-Value
Ion transport								
125	15235431	AT	Hydrogen ion transporting ATPase	14.2/6.1	182	114	−3.58	0.0067
128	15235431	AT	Hydrogen ion transporting ATPase	14.2/6.1	253	203	−7.57	0.0013
156	56693623	BN	Porin-like protein	29.4/9.01	290	252	2.19	0.0370
Unknown								
37	15241808	AT	Hypothetical protein	54.0/5.0	120	97	2.12	0.0029
120	15241645	AT	Unknown protein	37.3/5.9	150	121	1.81	0.0072
Miscellaneous								
62	30680831	AT	Vesicle-associated membrane family protein	43.5/6.5	230	185	3.43	0.0054
97	17807	BN	Cruciferin cru4 subunit	20.3/8.9	197	99	1.18	0.0024
99	17807	BN	Cruciferin cru4 subunit	20.3/8.9	414	352	−1.26	0.0031
150	15237646	AT	Carbonic anhydrase family protein	31.4/7.14	254	202	−8.41	0.0048
153	158564071	AT	Cysteine-rich repeat secretory protein 26 precursor	30.3/9.15	150	110	−2.17	0.0021
59	14600070	BJ	S-adenosylmethionine synthetase	42.0/5.8	238	178	2.12	0.0043

No significant matches were found for the spot numbers not listed in the table

AT *Arabidopsis thaliana*; BC *Brassica carinata*; BJ *Brassica Juncea*; BN *Brassica napus*; BO *Brassica oleracea*; BR *Brassica rapa*; LP *Lolium perenne*; NT *Nicotiana tabacum*; OS *Oryza sativa*

^a Theoretical molecular weight and pI of the identified proteins

^b Mascot score for MS data

^c Mascot score for MS/MS data

^d Spot volume ratio between germinating (GP) and mature pollen (MP)

growth and cell wall synthesis, place a high demand on energy and carbon reserves. It is therefore critical for pollen grains to have these reserves in place at the time of maturity so that they are available for successful germination and tube growth. In this study, we have shown that mature canola pollen grains contain a large complement of proteins, the majority of which belonged to carbohydrate and energy metabolism, protein metabolism and cell wall remodeling groups (Table 1, and Figs. 2, 4a). Other proteomic studies have shown that the same groups of proteins also predominate in the mature pollen of rice (Dai et al. 2006), tomato (Sheoran et al. 2007), *Arabidopsis* (Holmes-Davis et al. 2005; Noir et al. 2005; Sheoran et al. 2006), tea (Li et al. 2008), and two species of gymnosperm (Fernando 2005; Chen et al. 2006). Together, these studies confirm that, as for pollen transcripts (Becker et al. 2003; Honys and Twell 2003; Pina et al. 2005), mature pollen store a large number of pre-synthesized proteins which could provide the immediate resource for rapid germination and early tube growth. Recent study on tobacco pollen has shown the presence of ribonucleoprotein particles (EPP) complexes (which contain rRNAs and mRNAs) in mature pollen and are highly activated during pollen germination indicating their role in protein synthesis, processing and transport in tube growth (Honys et al. 2009).

Mature pollen grains are desiccated structures (Taylor and Hepler 1997; Shivanna 2003), and are metabolically quiescent when released from the anthers. Upon landing on the stigma, pollen grains absorb moisture and become metabolically active. Several studies have investigated the physiological and biochemical changes (Mascarenhas 1993; Hepler et al. 2001; Shivanna 2003; Malhó 2006; Krichevsky et al. 2007) and signaling networks (Cheung and Wu 2008) associated with pollen germination and tube growth. In this study, we focused on analyzing the dynamics of protein expression during pollen germination in canola. Although the 2-DE protein profiles of MP and GP appeared similar (Figs. 2, 3), 344 protein spots showed twofold or greater differences in intensity between the two samples, based on DIGE image analysis. This is a much higher number than that reported for differentially expressed proteins in germinating rice and *Pinus* pollen. In rice, 186 protein spots showed differential expression (Dai et al. 2007a) whereas in *Pinus strobus*, 57 protein spots were more intense, or were only observed, in germinating pollen gels (Fernando 2005). The greater number of differentially expressed proteins observed in our study could be due to the use of DIGE technology, which is more sensitive than the CCB staining used in other studies.

Fig. 4 **a** A pie chart of functional groups of the differentially expressed proteins in mature and germinating pollen of canola. **b** The number of proteins up-regulated in mature (MP) and germinating pollen (GP) in different functional groups

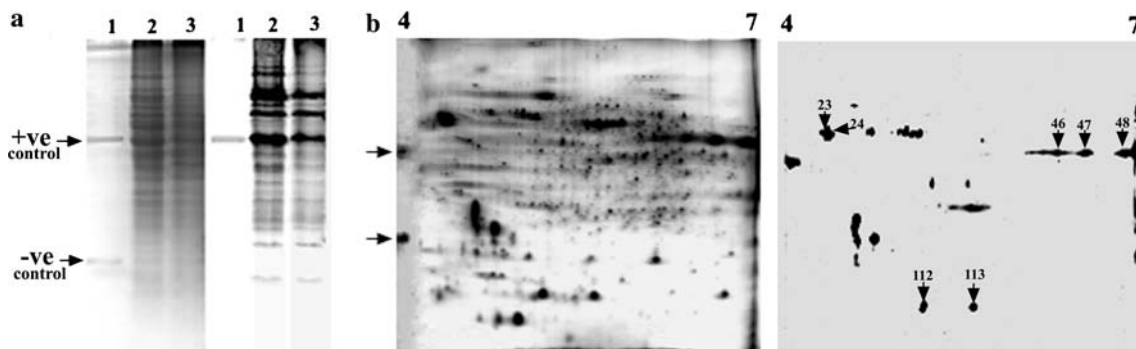
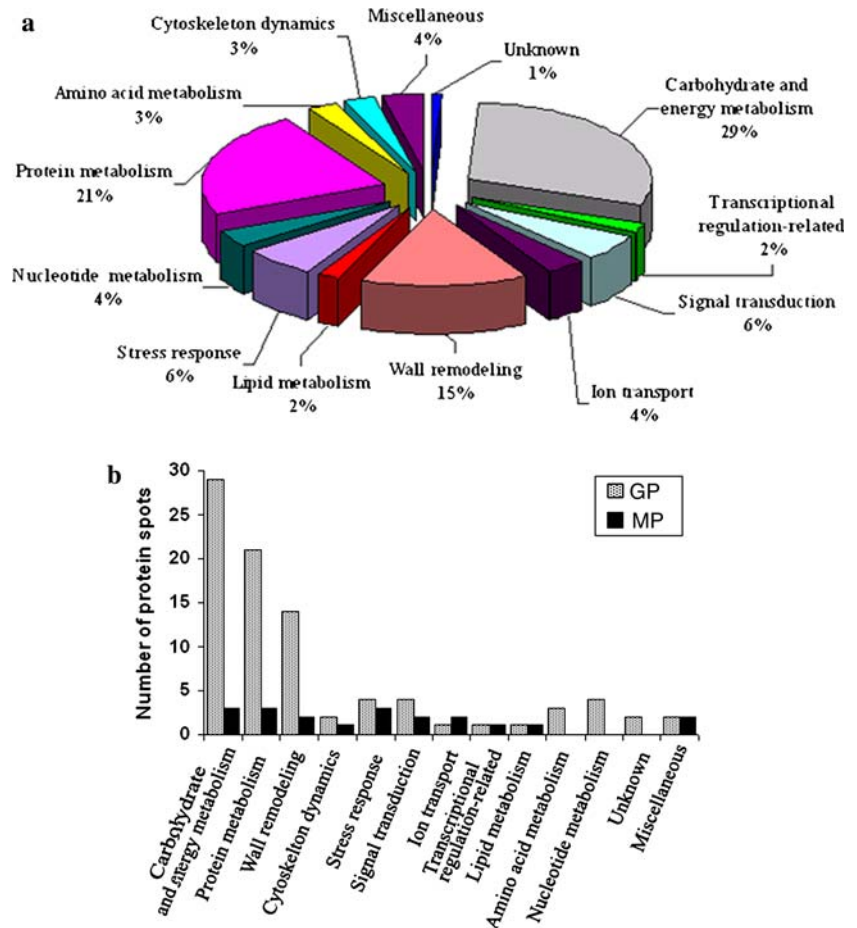


Fig. 5 **a** A 1-DE gel of mature (lane 2) and germinating pollen (lane 3) of canola. The left panel was stained with CCB for total protein and the right panel with Krypton for glycoprotein staining. Lane 1 represents controls for glycoprotein: +ve control (horseradish peroxidase),

–ve control (soybean trypsin inhibitor). **b** 2-DE gels from mature pollen of canola stained with CCB (left) and Krypton glycoprotein stain (right). The numbered spots matched the spots in Fig. 2

Of the proteins identified in mature canola pollen, 37 were related to energy metabolism, of which 29 were up-regulated in GP (Table 1, Fig. 4b). Some of these proteins belong to the glycolytic pathway (e.g., aldolase, enolase, triose-phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and phosphoglycerate mutase), TCA-cycle (e.g., aconitate hydratase, MDH, and NADH dehydrogenase), or electron transport chain (e.g., ATPase

and cytochrome *b5* reductase). Up-regulation of these proteins in GP is consistent with active energy and carbon metabolism during pollen germination and early tube growth. Proteins involved in energy metabolism were also observed during pollen germination in rice and *Pinus* (Dai et al. 2007b). In addition, we observed three isoforms of inorganic pyrophosphatase (IPP, a highly conserved enzyme which provides the driving force for many

metabolic reactions) that were not observed in germinating rice and *Pinus* pollen but which could be useful for energy metabolism during pollen germination. High activity of IPP and its phosphorylated form have been shown to regulate pollen tube growth and self-incompatibility in *Papaver* sp. (de Graaf et al. 2006). Many of the energy-related proteins are known to have multiple roles in plants. For example, GAPDH has been implicated in signaling (Hancock et al. 2005) and enolase in stress tolerance (Lee et al. 2002). Thus, some of the glycolytic proteins identified in canola pollen may have other functions like signaling, microtubule binding, and transcriptional activation, as observed in animal cells (Sriram et al. 2005).

Peroxisomal MDH (two isoforms) and 3-ketoacyl-CoA thiolase, which are involved in beta-oxidation of fatty acids, were also identified in canola pollen, and were highly expressed in GP compared to MP (Table 1). Mutant analyses have shown that, in addition to its established role in lipid catabolism during oil seed germination, beta-oxidation also plays a significant role in intracellular signaling (Baker et al. 2006). These enzymes, which were not reported in pollen germination of other species (Dai et al. 2007b), may therefore have a role in both fatty acid metabolism and cell signaling during canola pollen germination.

Aldolase is physically associated with vacuolar H⁺-ATPase in yeast (Lu et al. 2004) and rice roots (Konishi et al. 2005) and is thought to have a regulatory role in cell elongation. Ion gradient fluxes, particularly those of Ca²⁺ and H⁺, have been documented in tobacco pollen (Malhó et al. 2006; Certal et al. 2008) and are essential for the polarized and rapid growth of pollen tubes (Holdaway-Clarke and Hepler 2003; Cheung and Wu 2008). In canola pollen, a low molecular weight H⁺-transporting ATPase (two spots #s 125 and 128) was identified in MP and in PGM (Table 1, Fig. 2) indicating that this protein is released from pollen grains during germination and may have a role in cell elongation and polarized pollen tube growth. We also identified Ca²⁺-binding proteins (six spots) in canola pollen, which have an established role in pollen germination (Malhó et al. 2006). Only one Ca²⁺-binding protein was reported in rice pollen, and it was down-regulated during germination (Dai et al. 2007a).

Several proteins associated with cell wall remodeling and wall metabolism were also identified in canola pollen, including UDP-glucose pyrophosphorylase, polygalacturonase (PG), reversibly glycosylated polypeptide-1, leucine-rich repeat family/extensin family protein, pollen-specific protein Bnm1, β -galactosidase, glycoside hydrolase family 28 protein, pectinesterase inhibitor, and pollen allergen Ole e 1 (Table 1). Of these, 14 proteins showed higher accumulation in GP relative to MP (Table 1, Fig. 4b), which is indicative of their involvement in cell wall formation in growing pollen tubes. UDP-glucose pyrophosphorylase,

UDP-glucose dehydrogenase, and reversibly glycosylated polypeptide-1 are known to be the key proteins involved in cell wall polysaccharides biosynthesis in pollen development (Drakakaki et al. 2006; Schnurr et al. 2006). Some of these proteins were also reported in germinating pollen of rice and *Pinus* (Dai et al. 2007a; Fernando 2005).

One of the highly abundant proteins identified in canola pollen was polygalacturonase (PG), also known as pectinase, with three of the seven isoforms identified and showed higher accumulation in GP relative to MP (Table 1). Pollen-specific PG genes have been reported in various species, including *B. napus* (Robert et al. 1993; Dearnaley and Daggard 2001). PG belongs to a family of glycosyl hydrolases that digest the 1, 4- α -D-galactosiduronic linkages in pectate and other galacturonans, and are known to function during pollen germination and/or tube growth by softening of the cell wall (Minic 2008). PG may also have a role in the degradation of the walls of stigmatic/stylar cells to allow penetration of the pollen tube, since this protein was secreted into the PGM during in vitro germination (Fig. 3). In rice, three isoforms of PG were down-regulated in germinating pollen compared to mature pollen (Dai et al. 2007a).

The pollen-specific protein Bnm1 identified in canola pollen was another abundant protein, with four isoforms identified. The *Bnm1* gene is specifically expressed in *B. napus* pollen (Treacy et al. 1997), and this protein, which has a pectin methyl esterase inhibitor (PMEI) conserved domain, was not reported in mature or germinating pollen of other species (Fernando 2005; Dai et al. 2006, 2007b; Chen et al. 2006; Sheoran et al. 2006, 2007; Li et al. 2008). Pectin constitutes a major component of plant cell walls and its metabolism is essential for the rapid pollen tube growth. Pectin methyl esterase (PME), PG, and PMEI have been implicated in pectin metabolism in pollen germination and tube growth (Bosch and Hepler 2006; Krichevsky et al. 2007). PME and PMEI were also reported in mature pollen of rice (Dai et al. 2007a), Arabidopsis (Sheoran et al. 2006), and tomato (Sheoran et al. 2007), but a change in their expression was not reported during rice pollen germination (Dai et al. 2007a).

Another enzyme involved in cell wall metabolism, and over-expressed in GP of canola, was β -galactosidase (two isoforms, Table 1). This enzyme hydrolyzes the terminal galactosyl residues from carbohydrates, arabinogalactan, glycoprotein and galactolipids, and has a role in the degradation of cell wall components during cell wall expansion, fruit ripening, senescence, and storage mobilization. This enzyme was reported in lily pollen (Singh and Knox 1985) and was associated with pollen development and subsequent germination in tobacco (Hrubá et al. 2005), although it was not identified in the germinating pollen of rice or *Pinus* (Fernando 2005; Dai et al. 2007a). A leucine-rich

repeat family protein with roles in cell wall development, including that of pollen tubes (Rubinstein et al. 1995), was identified in canola pollen and showed higher expression in GP compared to MP (Table 1).

The cytoskeleton is an essential component of tip growth in plants, especially the pollen tube (Cai et al. 1997; Malhó et al. 2006). We identified actin and two isoforms of profilin (an actin-binding protein) in MP and GP of canola and their levels increased during pollen germination (Table 1). These proteins have an established role in pollen tube growth (Taylor and Hepler 1997; Malhó 2006) and their presence was reported in most proteomic studies of pollen (Sheoran et al. 2006, 2007; Dai et al. 2007b). Vidali and Hepler (1997) did not find any change in the level of profilin in germinating lily pollen, although an increase in profilin 1 and decrease in profilin 3 was reported in hydrated lily pollen (Miki-Hirosige et al. 2004).

Protein synthesis and metabolism are important factors in pollen germination and tube growth; however, the requirement for protein synthesis varies with species (Mascarenhas 1993; Taylor and Hepler 1997). In mature canola pollen, we identified three proteins involved in protein synthesis, i.e., eukaryotic translation initiation factor-5A and two elongation factors, and they were up-regulated in GP (Table 1). Eighteen proteins involved in protein folding, processing, and degradation including, HSPs, BIPs, chaperones, cell division control (CDC) proteins, peptidases, RPT5A, protein binding/ubiquitin-protein ligase, and proteasome subunits were also up-regulated in GP, while phytolectin and prohibitin 1-like protein were down-regulated in GP, compared to MP (Table 1, Fig. 4b). Interestingly, one isoform of peptidyl-prolyl *cis-trans* isomerase showed increased levels of expression during germination while another showed decreased levels (Table 1). These results are reflective of the importance of rapid protein turnover (Sheng et al. 2006) and proper folding in pollen germination and early tube growth. In tobacco mature and germinating pollen, a number of proteins related to protein synthesis, processing and localization were identified (Honys et al. 2009). In contrast, relatively few proteins involved in protein synthesis, degradation and folding were identified during pollen germination in rice (Dai et al. 2007b).

Pollen grains are subjected to various biotic and abiotic stresses when released from the anther. A number of proteins associated with stress response were identified in mature canola pollen, including catalase, peroxidase, GRP and LEA proteins (Table 1). Two isoforms of catalase, peroxidase and GRP were up-regulated, and two isoforms of catalase and LEA protein down-regulated, in GP (Table 1). Catalase and peroxidase are known to regulate H_2O_2 , a toxic metabolite in tissues. However, H_2O_2 is also known to modulate Ca^{2+} -mobilization in plants (Neill et al. 2002)

and, therefore, catalases and peroxidases might have dual roles in stress tolerance and calcium signaling during canola pollen germination and tube growth. The presence of catalase was, however, not reported in rice and *Pinus* pollen tube development (Fernando 2005; Dai et al. 2007a). GRPs are known to have a role in post-transcriptional regulation of genes, but also in defense against abiotic stresses (Kim et al. 2005). LEA proteins are known to provide desiccation tolerance (Park et al. 2005) which, although important in mature pollen, may no longer be required in hydrated germinating pollen. GRPs and LEA proteins have also been identified in the mature pollen of other species (Sheoran et al. 2006, 2007).

When pollen grains land on the stigma of a flower they release various proteins (Taylor and Hepler 1997; Shivanna 2003), either from the pollen coat or pollen wall (Suen et al. 2003; Dai et al. 2006), which have multiple roles in pollen–stigma interaction, pollen germination, early tube growth and self-incompatibility. During in vitro germination of canola pollen a number of proteins were released into the PGM and this correlated with down-regulation of the same proteins in MP (Fig. 3). These included cruciferin, oleosin, cysteine-rich repeat secretory protein 26 precursor, enolase, and one of the isoforms of inorganic pyrophosphatase, all of which were more abundant in the PGM than in MP. Several other proteins, including PG and pectinesterase inhibitor, were also released into the PGM during germination. Oleosin, one of the major coat protein, and cruciferin, a storage protein, were reported in *Brassica* pollen (Sjodahl et al. 1991; Ross and Murphy 1996). The release of these and other proteins in the PGM during pollen germination suggests their possible role in pollen–stigma interaction and early tube growth. Based on the analysis of water-washed pollen, 41 proteins were identified as potentially being released from rice pollen, although the authors were unable to isolate proteins from the germination medium (Dai et al. 2007a).

In conclusion, this study documents the presence of a large number of proteins and enzymes in the mature pollen of *B. napus*. Using the DIGE technology, we have identified a number of proteins that were up-regulated, and a few down-regulated, during pollen germination and early tube growth. Many of the up-regulated proteins have designated roles in energy metabolism, protein processing, cell wall modeling, Ca^{2+} -signaling, ion transport, and cytoskeleton formation, all of which are important for pollen germination and tube growth. The down-regulated proteins included stress-related LEA proteins, which may not be critical after pollen hydration. Analysis of the pollen germination medium showed that some proteins are secreted from the pollen during germination, and they may have a role in pollen–stigma interaction and early tube growth. The results of this study provide unique insights into the

dynamics of protein expression during pollen germination and early tube growth, processes that are vital for successful fertilization in seed plants.

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