

REGULAR ARTICLE

Characterization of pollen tube development in *Pinus strobus* (Eastern white pine) through proteomic analysis of differentially expressed proteins

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The differentially expressed proteins in pollen tubes indicate their specific roles in this stage of male gametophyte development. To isolate these proteins, 2-DE was done using ungerminated pollen and 2-day-old pollen tubes of *Pinus strobus*. Results show that 645 and 647 protein spots were clearly resolved from pollen grains and pollen tubes, respectively. Thirty-eight protein spots were expressed only in pollen tubes, while 19 increased in intensity. MALDI-TOF MS was used to generate tryptic peptide masses that were submitted to Mascot for identification. Of the differentially expressed proteins, 12% matched with hypothetical proteins, 33% did not hit any protein, and for the 55%, a putative function was assigned based on similarity of sequences with previously characterized proteins. Therefore, pollen tube development can be characterized by the cellular activities that involve metabolism, stress/defense response, gene regulation, signal transduction, and cell wall formation. This study expands our understanding of the changes in protein expression associated with pollen tube development and provides insights into the molecular programs that separate the development of the pollen tubes from pollen grains. This is the first report that describes a global analysis of differentially expressed proteins from the pollen tube of any seed plant.

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1 Introduction

Pollen tube development in pines differs from those of flowering plants in various ways. This includes a slow rate and extended period of growth, an extremely delayed gametogenesis, a wall that is made up of cellulose rather than callose, a branched pattern, absence of organelle zonation, and distinct cytoskeletal control. These differences are not subtle but represent major evolutionary divergence in the development of the male gametophytes in

seed plants. Therefore, a different mechanism of pollen tube development may be in place in pines, and this warrants a deeper investigation. Of the gymnosperms, the pines have been established as the model system for studying plant evolution, wood formation, and perennial growth [1]. In addition, pines produce unlimited quantities of pollen grains, which can be obtained free from contaminants and, therefore, ideal in studies requiring a lot of pollen tubes [2–4].

Pine pollen tubes play a central role in sexual reproduction by delivering the male gametes into the egg cytoplasm. Critical to this role is the growth and development of the pollen tubes; a subject area less understood than that in flowering plants. There are many reports on proteins associated with pollen tube development in flowering plants, which include kinases [5, 6], proteasome [7], Tyr phosphatase [8], profilin [9], pectin methylesterase [10], calmodulin and

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cAMP [11], Ca²⁺ ATPases [12], and cyclophilin [13]. However, it is not known if any of these are also expressed in the pollen tubes of gymnosperms. Global analyses of proteins expressed in various cells and tissues have been done, e.g., for pine needles and xylem [14], poplar xylem [15], wheat-grain endosperm [16], rice anthers [17], *Arabidopsis* cell walls [18], and tobacco BY2 cells [19]. High-throughput protein analysis has been applied to examine the development of germinating seeds of *Arabidopsis* [20] and barley [21], and various stages of rice male gametophyte [22]. However, there are no reports available on the global analysis of proteins expressed in the pollen tubes from any seed plant.

Although proteomic analysis has been performed on rice using anthers containing young microspores [17] or developing male gametophytes at various stages of development [22], these reports did not discriminate between the proteins expressed in the gametophytic and sporophytic tissues of the anther. During the development of the male gametophytes, anther tissues such as the epidermis, endothecium, middle layers, and tapetum undergo various developmental changes, certainly with corresponding changes at the protein level [23]. Therefore, a study that is truly representative of the proteome of the male gametophyte is still lacking.

This study is aimed at identifying the proteins that are expressed in the pollen tubes but not in the pollen grains, and the proteins which are upregulated during pollen tube development. The results will point toward interesting proteins that can be pursued for further analysis of pollen tube development. This study will provide insights into the changes in gene expression associated with the formation of pollen tubes and the molecular programs that define this critical stage of sexual reproduction in seed plants.

2 Materials and methods

2.1 Plant material

Pollen cones of *Pinus strobus* (Eastern white pine) were collected from three different trees growing at the Lafayette Experimental Station of the State University of New York College of Environmental Science and Forestry. Pollen cones were collected 1–2 days prior to dehiscence of the microsporangia. The cones were decontaminated by washing with freshly prepared 70% ethanol, sterile distilled water, and 1% sodium hypochlorite, each step for 30 s. The cones were then rinsed three times with sterile distilled water, each step for 10 s. They were blotted dry on sterile paper towels and put in sterile glass petri dishes lined with sterile filter paper. Petri dish covers were replaced with sterile filter papers and fastened with rubber bands to ensure sterility during drying of the pollen cones and shedding of pollen grains. The cones were allowed to release the pollen grains by incubating at 25°C for 3 days. Dried sterile pollen grains were collected and stored in sterile vials at –20°C.

2.2 Media composition and culture conditions

The pollen germination medium contained 0.1 mg/mL H₃BO₃, 0.3 mg/mL Ca(NO₃)₂, 0.2 mg/mL MgSO₄, 0.1 mg/mL KNO₃, supplemented with 145 mM sucrose and 0.3% phytigel. The pH was adjusted to 5.8. Three pieces of sterile nylon membranes (Gelman Sciences, Ann Arbor, MI, USA) were placed on the solidified pollen germination medium and pollen grains were evenly dispensed by gently shaking a sterile spatula containing pollen grains over them. Approximately 50 mg pollen grains were evenly distributed over the three nylon membranes. The pollen grains were allowed to germinate by incubation in the dark at 27°C for 2 days.

2.3 Protein extraction

Pollen grains (50 mg) taken directly from the freezer and 2-day old pollen tubes which were scraped off from the nylon membranes, were immediately frozen with liquid nitrogen, grounded into a fine powder, and collected into a sterile centrifuge tube containing 750 µL protein extraction buffer [65 mM Tris, 1% SDS, 5% glycerol, 2.5% 2-mercaptoethanol, and 7.5 µL protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), pH 6.8]. The suspension was vortexed at high speed for 2 min, boiled for 5 min and frozen at –80°C for 1 h. The suspension was thawed, boiled for 5 min, and centrifuged at 14 000 × g for 15 min at 4°C. The supernatant was carefully transferred into a new centrifuge tube. The total soluble protein extract was stored at –80°C until use. The concentrations of proteins were estimated using the Bradford Protein Assay kit (Bio-Rad, Hercules, CA, USA) with a range of known concentrations of BSA as standards.

2.4 Gel electrophoresis

2-DE was performed according to O'Farrell [24]. Briefly, in the first dimension, IEF was carried out in glass tubes (2.0-mm id) with 2% pH 4–8 ampholine (Pharmacia, Baltimore, MD, USA) for 9600 Vh. The tube gel was calibrated by adding 50 ng tropomyosin to each 100 µL protein sample prior to loading. Tropomyosin is an IEF standard that shows two polypeptide spots of similar pI. The lower spot (33 kDa, pI 5.2) is marked with an arrowhead on the tube gels. After equilibration for 10 min in buffer "0" (10% glycerol, 50 mM DTT, 2.3% SDS, and 0.0625 M Tris, pH 6.8), each tube was sealed to the top of a stacking gel that overlaid a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 5 h at 25 mA/gel. High molecular mass standard markers (14–220 kDa) (Bio-Rad) were used and appeared on the basic side of the special silver-stained gels. In special silver staining, the use of glutaraldehyde was omitted and the protocol followed was that of Shevchenko [25]. Special silver staining was used because of its very high sensitivity and compatibility with MS [25–27]. The gels were dried between transparent cellulose sheets.

2.5 Gel comparison and analysis

Triplicate silver-stained 2D gels from three separate protein extractions obtained from three different individuals were analyzed. Only those protein spots that were consistently resolved from the three gels were considered in the analysis. A laser densitometer (PDSI, Molecular Dynamics Inc., Sunnyvale, CA, USA) was used to scan the gels. The images were analyzed using Progenesis Discovery Software (Nonlinear Dynamics, Durham, NC, USA) such that all major spots were outlined, quantified and matched on all of the gels. Computerized analysis of the gels included parameters such as automatic spot finding, quantification, automatic background subtraction, and automatic spot matching in conjunction with detailed manual examination of the spots.

2.6 MS

The 57 protein spots that were determined to be differentially expressed (unique or upregulated in pollen tubes) were individually and manually excised from the dried gels. They were rehydrated, washed, reduced with DTT (10 mM in 100 mM NH_4CO_3), alkylated with iodoacetamide (55 mM in 100 mM NH_4CO_3), and rinsed. The proteins were digested in 50 μL 10 mM Tris-HCl (pH 8.0) containing 1 μM trypsin (Sigma) at 37°C for 10–12 h. The peptides were extracted with 25 mM NH_4CO_3 , ACN and 5% formic acid. The peptides were purified from the extracts using C18 ZipTips (Millipore, Bedford, MA, USA).

The purified peptides (2 μL) were added directly to the matrix solution (0.1 mg/mL CHCA, 90% ACN, 0.1% TFA, and 9.9% deionized distilled water), spotted onto a Mass-REP PROtarget plate (Waters, Milford, MA, USA), air dried, rinsed, and tryptic peptide masses were obtained using a ToF-Spec 2E MALDI-TOF mass spectrometer (Waters). The set of spectra generated were calibrated internally with tryptic autodigest ions (842.51 and 2211.10 Da) and externally using sequazyme calibration mixture 1 containing des-arg-bradykinin, angiotensin 1, glufibrinopeptide B, and neurotensin (Applied Biosystems, Foster City, CA, USA). Keratin and porcine trypsin peak masses were put on an exclude list to prevent these ions from being selected during analysis.

Matching of experimental tryptic digests with theoretical digests of the proteins in the database was performed using the MASCOT interface [28]. The nonredundant NCBI and Swiss-Prot databases were searched. A conservative search was done by restricting the taxonomic category to green plants (*Viridiplantae*). Queries were carried out taking into consideration the experimental molecular mass and pIs of individual proteins, and possible post-translational modifications such as oxidation of methionine and modification of cysteine by acrylamide. The maximum number of missed cleavages was set at one. Mass accuracy was set at ± 100 ppm with a minimum requirement of five peptides matched. The confidence of the matches was also based on the percentage

sequence coverage. Based on all these parameters, only the match that appeared at the top of the list was considered as positive identification.

3 Results

3.1 Proteome maps of pine pollen grains and tubes

Analysis of the triplicate silver-stained 2-D gels showed that 645 and 647 protein spots were clearly and consistently resolved from pollen grains and tubes of *P. strobus*, respectively. These proteins were in the pI range of 4–8 and molecular mass range of 14–220 kDa. Thirty-six protein spots in pollen grains were not found in pollen tubes (data not shown), while 38 new protein spots appeared during pollen tube development (Fig. 1). These differentially expressed proteins represent about 6% of the total proteins in both pollen grains and tubes.

Pollen grains and tubes expressed the same 609 protein spots, which represent about 94% of the total proteins in these structures. When the expression levels of these common proteins were compared between the two developmental stages, 19 protein spots showed at least threefold increase in the pollen tube (Fig. 2), while 52 protein spots showed at least threefold decrease in the pollen tube (data not shown).

3.2 Protein identification through cross-species matching

Fifty-seven protein spots were processed by in-gel trypsin digestion and MALDI-TOF MS. The spectra generated were searched against the nonredundant NCBI and Swiss-Prot databases. The taxonomic category searched was restricted to the green plants (*Viridiplantae*). Based on cross-species matching, the putative identities of the proteins expressed only in the pollen tubes of *P. strobus* are presented in Table 1. Table 2 shows the putative identities of the proteins in *P. strobus* that showed at least threefold increase in staining intensity in the pollen tubes.

3.3 Functional categories of differentially expressed proteins

Seven of the 57 protein spots (12%) that were analyzed through MALDI-TOF MS matched with hypothetical proteins, while 19 protein spots (33%) did not match with any protein in the databases that were searched. For 31 of the differentially expressed proteins (55%), a putative function can be assigned based on their similarity with previously characterized proteins, which were mostly from other species. To facilitate the interpretation of the results, all of the differentially expressed proteins were combined and grouped into their respective functions (Fig. 3). The results show that the differentially expressed proteins in the pollen

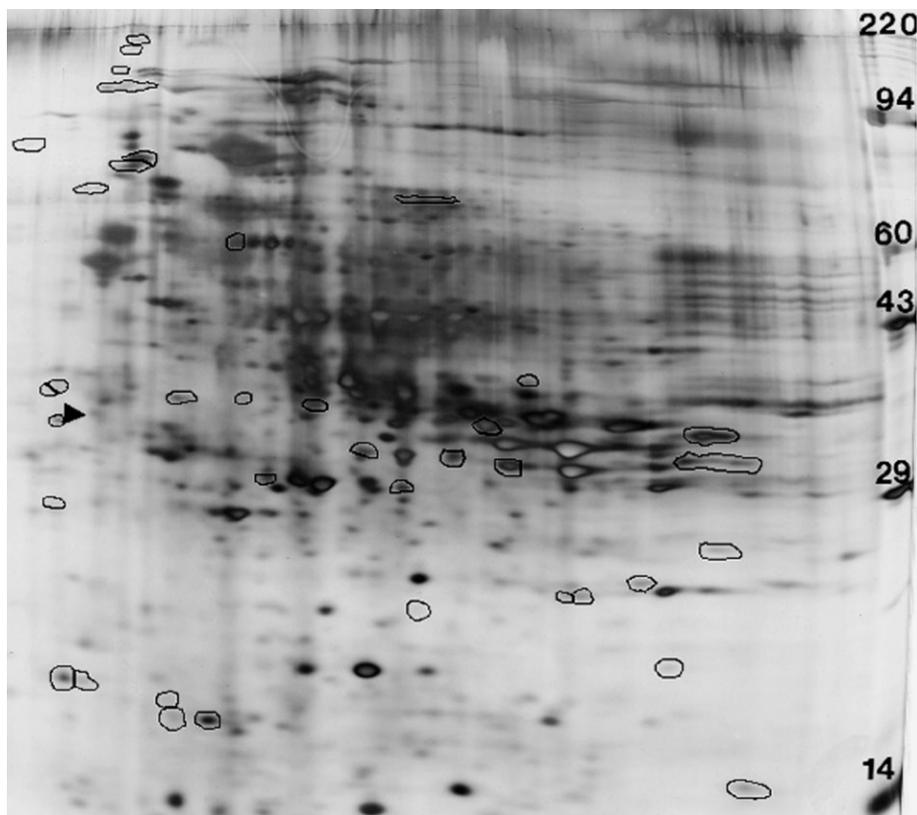


Figure 1. Proteins expressed in *P. strobus* pollen tubes but not in pollen grains.

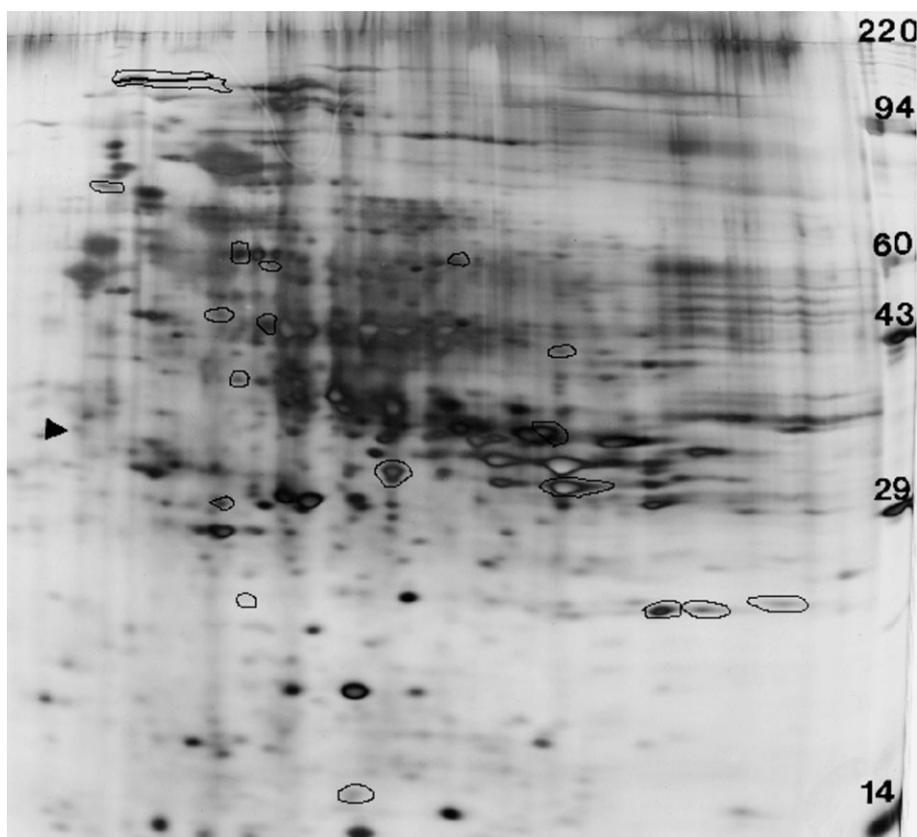


Figure 2. Proteins with at least threefold increase in staining intensity in *P. strobus* pollen tubes.

Table 1. Putative identities of proteins expressed in the pollen tubes but not in the pollen grains of *P. strobus*

Spot no.	Mol. mass/pI	Matched proteins	Accession no.	Matched species	Functional category	Matched peptides/% coverage
646	78/4.97	Hypothetical protein	Q80920	<i>Arabidopsis thaliana</i>	Unknown function	6/33
647	172/5.35	Hypothetical protein	Q9C5M6	<i>Arabidopsis thaliana</i>	Unknown function	10/12
649	69/5.19	No match	–	–	No match	–
651	26/7.45	α -expansin	Q9SWY1	<i>Pinus taeda</i>	Cell wall biosynthesis	7/30
652	14/7.50	ADP-glucose pyrophosphorylase small subunit	Q9FUJO	<i>Solanum tuberosum</i>	Metabolism	9/29
653	20/7.30	Cyclophilin	Q8VXA5	<i>Pseudotsuga menziesii</i>	Stress/defense response	9/21
656	35/5.77	Putative nucleic acid binding protein	Q94LL0	<i>Oryza sativa</i>	Gene regulation	5/21
657	34/5.05	No match	–	–	No match	–
658	34/5.02	No match	–	–	No match	–
659	32/5.10	UDP-glucose pyrophosphorylase	Q43772	<i>Hordeum vulgare</i>	Metabolism	9/30
660	18/5.49	No match	–	–	No match	–
661	32/6.73	Isoflavone reductase	P52578	<i>Medicago sativa</i>	Stress/defense response	5/17
662	159/5.35	No match	–	–	No match	–
663	134/5.27	F-box family protein-like	XP465719	<i>Oryza sativa</i>	Gene expression	7/39
664	111/5.30	No match	–	–	No match	–
665	77/5.37	Hypothetical protein	Q8RWB1	–	Unknown function	5/14
666	74/5.32	ADP-glucose pyrophosphorylase large subunit	Q22658	<i>Citrullus lanatus</i>	Metabolism	9/26
667	56/5.73	Enolase	Q43321	<i>Alnus glutinosa</i>	Metabolism	8/56
668	71/6.13	No match	–	–	No match	–
669	38/6.76	Calcium-dependent protein kinase	Q9LJL9	<i>Arabidopsis thaliana</i>	Signal transduction	6/18
670	34/6.62	Fructose-bisphosphate aldolase	Q8LK59	<i>Metasequoia glyptostroboides</i>	Metabolism	7/20
671	32/6.51	Phenylcoumaran benzylic ether reductase	Q9LDB5	<i>Tsuga heterophylla</i>	Stress/defense response	5/16
672	31/7.50	Malate dehydrogenase precursor	Q48904	<i>Medicago sativa</i>	Metabolism	8/43
673	34/7.42	Putative receptor kinase	Q9FE99	<i>Oryza sativa</i>	Signal transduction	7/24
674	24/7.15	Glutamine synthetase	Q42688	<i>Chlamydomonas reinhardtii</i>	Metabolism	12/40
675	24/6.87	Enhancer-of-zeste protein	Q84UI6	<i>Oryza sativa</i>	Gene regulation	21/31
676	24/6.94	Ferredoxin-thioredoxin reductase	P41347	<i>Zea mays</i>	Metabolism	5/46
677	38/5.99	Hypothetical protein	Q9SNF2	<i>Oryza sativa</i>	Unknown function	7/25
678	32/6.21	Phenylcoumaran benzylic ether reductase	Q9LL41	<i>Pinus taeda</i>	Stress/defense response	5/16
679	31/6.32	Major surface-like glycoprotein	Q67ZD0	<i>Arabidopsis thaliana</i>	Signal transduction	10/16
680	36/5.52	Auxin-induced protein 2	Q7XYT5	<i>Pinus taeda</i>	Gene regulation	5/36
681	30/5.82	Ascorbate peroxidase	Q9FPF1	<i>Pinus strobus</i>	Stress/defense response	11/32
682	17/5.48	Hydroperoxide lyase	Q9STA2	<i>Medicago sativa</i>	Stress/defense response	14/51
683	17/5.6	Remorin-like protein	XP466591	<i>Oryza sativa</i>	Signal transduction	7/37
685	23/6.34	No match	–	–	No match	–
686	27/5.09	Hypothetical protein	Q8LE16	<i>Arabidopsis thaliana</i>	Unknown function	8/31
687	16/5.18	No match	–	–	No match	–
688	16/5.10	No match	–	–	No match	–

Table 2. Putative identities of proteins upregulated (with at least threefold increase in staining intensity) in the pollen tubes of *P. strobus*

Spot no.	Mol. mass/pI	Matched proteins	Accession no.	Matched species	Functional category	Matched peptides/% coverage
5	138/5.40	No match	–	–	No match	–
7	129/5.40	Dihydrokaempferol 4-reductase	P93777	<i>Sorghum bicolor</i>	Stress/defense response	6/23
50	72/5.34	No match	–	–	No match	–
100	58/6.57	No match	–	–	No match	–
110	55/5.79	ATP synthase β -subunit	P17614	<i>Nicotiniana plumbaginifolia</i>	Metabolism	11/28
128	52/5.90	Hypothetical protein	Q75H74	<i>Oryza sativa</i>	Unknown function	5/32
183	42/5.73	No match	–	–	No match	–
191	42/5.89	Alcohol dehydrogenase	Q43022	<i>Pinus banksiana</i>	Metabolism	12/22
202	46/6.93	No match	–	–	No match	–
259	38/5.78	No match	–	–	No match	–
293	35/6.90	Putative gag-pol polyprotein	Q6L3Y6	<i>Solanum demissum</i>	Gene regulation	10/12
355	32/6.35	Phenylcoumaran benzylic ether reductase	Q9LDB5	<i>Tsuga heterophylla</i>	Stress/defense response	5/15
358	32/6.96	Phenylcoumaran benzylic ether reductase	Q9M525	<i>Tsuga heterophylla</i>	Stress/defense response	5/15
404	30/5.75	No match	–	–	No match	–
465	24/7.65	At3g18730	Q6Q4D0	<i>Arabidopsis thaliana</i>	Gene regulation	12/15
470	24/7.41	No match	–	–	No match	–
477	24/7.30	Hypothetical protein	NP189708	<i>Arabidopsis thaliana</i>	Unknown function	5/82
489	24/5.83	No match	–	–	No match	–
606	15/6.23	Putative steroid 22- α -hydroxylase	Q8H848	<i>Oryza sativa</i>	Signal transduction	11/30

tubes of *P. strobus* are involved in various aspects of metabolism (18%), stress/defense response (16%), gene regulation (10%), signal transduction (9%), and cell wall formation (2%).

4 Discussion

4.1 Proteins in pine pollen tubes

Through silver staining, the number of protein spots that were resolved in the pollen grains and tubes of *P. strobus* is quite similar to the number of proteins visualized in the xylem and needles of *P. pinaster* [14]. Unfortunately, there is no report available that described the number of protein spots only in the male gametophytes and which has been stained with silver. However, in rice anthers containing developing pollen grains, 4300 protein spots were detected from silver-stained gels [17]. Silver staining is not only a very sensitive method of visualizing proteins, it is also compatible with MS, allowing identification of proteins expressed even at low levels [25–27, 29, 30].

Proteomic analysis has been established as a viable approach to identify proteins in species whose genomes have not yet been sequenced [28, 31]. This is a relatively rapid

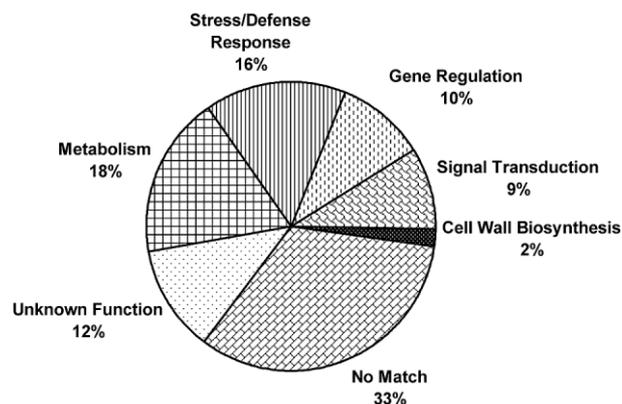


Figure 3. Functional categories of differentially expressed proteins in *P. strobus* pollen tubes.

approach that has allowed the identification of proteins from a variety of plants, including *P. pinaster* [14], *Populus trichocarpa* [15], *Triticum aestivum* [16], *Zea mays* [32], *Phleum pratense* [33], *Nicotiana tabacum* [19], *Hordeum vulgare* [21], *Cannabis sativa* [34], and even in a green alga, *Heamatococcus pluvialis* [35]. The genome of *P. strobus* has not yet been sequenced but proteomic analysis of differentially expressed

proteins in the pollen tubes revealed that approximately 55% of these proteins could be putatively identified by database search coupled with cross-species matching. Twelve percent of the differentially expressed proteins from *P. strobus* pollen tubes matched with hypothetical proteins, while 33% did not have any match in the NCBI and Swiss-Prot databases.

A very large fraction of the proteome of the mature ungerminated pollen is similar to the proteome of the pollen tube. This shows that 94% of the proteins in the pollen tubes are already present in the pollen grains even before they are dispersed from the cones. This supports previous reports that the protein profiles of germinated and ungerminated pollen are very similar, which is true in flowering plants [36, 37], as well as in pines [3, 38]. The very high correlation of protein profiles between these two developmental stages also suggests that the information obtained through the analysis of pollen grains would be very helpful in understanding what is also going on in the pollen tubes. In this regard, several reports have characterized the transcriptome of *Arabidopsis* pollen grains and expanded our knowledge of the number of pollen-expressed genes [39–41]. These reports have also identified several pollen-specific genes; however, the number varied considerably among the reports, *i.e.*, 10% [40], 40% [39], and 83% [41]. Nevertheless, the reports have shown that there is a large percentage of genes specifically expressed in the pollen grains. On the other hand, since proteins are the final products of the expression of genes, information on large-scale analysis of proteins from pollen grains and tubes is necessary to strengthen the data derived from transcriptome analysis. Unfortunately, it is currently difficult to obtain large quantities of *Arabidopsis* pollen grains for 2-D gel analysis [42].

The differentially expressed pollen tube proteins indicate their specific role in pollen tube development. However, there are hardly any reports available on the proteins involved in the reproductive development of gymnosperms. Therefore, most examples discussed in here pertain to what is known in flowering plants.

4.2 Proteins involved in metabolism

Mature pollen grains are known to contain large amounts of carbohydrates, which account for the major part of their total dry weight [43]. Developing pollen tubes have a very high metabolic activity and during pollen germination, carbohydrates are utilized as energy source to sustain pollen tube growth and development [44]. This proteomic analysis indicate that pine pollen tube development involves the expression of a suite of proteins engaged in carbohydrate metabolism, and these are expressed only in the pollen tubes (ADP-glucose pyrophosphorylase small subunit, ADP-glucose pyrophosphorylase large subunit, UDP-glucose pyrophosphorylase). UDP-glucose pyrophosphorylase is involved in sucrose biosynthesis [45]. The use of UDP-glucose is also coupled to the activity of ADP-glucose pyrophosphorylase, resulting in the production of ADP-glucose, which is the

only immediate precursor of starch in plants [46]. The activities of these enzymes suggest that sugars are also produced during the development of pine pollen tubes.

Pine pollen tubes express various enzymes which are involved in cellular energy metabolism (ATP synthase β -subunit, enolase, fructose-1,6-bisphosphate, malate dehydrogenase, ferredoxin:thioredoxin reductase and alcohol dehydrogenase) substantiating previous reports that this stage of male reproductive development is engaged in a very high metabolic activity. ATP synthase is the enzyme that synthesizes ATP from ADP and inorganic phosphate driven by a flux of protons across the membrane down the proton gradient generated by electron transfer. Enolase is a glycolytic enzyme that catalyzes the interconversion of 2-phosphoglycerate to phosphoenol pyruvate. Fructose-bisphosphate aldolase is also a glycolytic enzyme that catalyzes the reversible conversion of fructose-1,6-bisphosphate to dihydroxy-acetone-phosphate and glyceraldehydes-3-phosphate. Malate dehydrogenase is an enzyme of the tricarboxylic acid cycle that converts malate and NAD into oxaloacetate and NADH. NADH is fed into the electron transport chain to produce three molecules of ATP. Ferredoxin:thioredoxin reductase is an iron-sulfur protein, which is part of the electron transport system. Cellular respiration and alcoholic fermentation take place concurrently during high rates of sugar metabolism. The latter is facilitated by alcohol dehydrogenase and occurs to accommodate the increased demand for energy and biosynthetic intermediates, which are necessary for pollen development and germination [44].

One protein spot expressed only in pollen tubes matched with glutamine synthetase. This enzyme is the first catalyst in the pathway that brings nitrogen into cellular metabolism. Specifically, it is involved in the assimilation of ammonia generated in processes such as seed germination, photorespiration, nitrite reduction, nitrogen fixation, and assimilation from the soil [47].

4.3 Proteins involved in stress/defense response

This proteomic analysis has allowed the identification from pine pollen tubes of two of the three enzymes involved in the biosynthesis of phenylpropanoid-derived plant defense compounds, isoflavone reductase (1 protein spot) and phenylcoumaran benzylic ether reductase (4 protein spots). These enzymes are known to be involved in the biosynthesis of lignans, which act as defense against pathogens and oxidative stress [48]. Lignans also confer durability and longevity to cells. Pollen grains are non-aseptic as soon as they are released from the cones. Therefore, pollen grains also produce non-aseptic pollen tubes that penetrate between cells of the nucellus, which is wounded in the process. During this interaction, the production of defense compounds is crucial to protect not only the wounded nucellus from pathogens, but also the developing pollen tubes which remain partly germinated for quite sometime. Phenylcoumaran benzylic ether reductase probably provides protection to the develop-

ing pollen tubes against oxidative stress that occurs during the several months that they are embedded deep within the cones. Whereas this report points to the pollen tube as the source of isoflavone reductase, in *Solanum tuberosum*, it is produced by the female tissues in response to pollination [49].

Three protein spots which are expressed only in pine pollen tubes are involved in various stress responses (cyclophilin, ascorbate peroxidase, and hydroperoxide lyase). In *Tradescantia virginiana* and *Cryptomeria japonica* (a gymnosperm), cyclophilin is released from pollen when germination is suppressed [13]. The expression of cyclophilin is regulated by a variety of environmental stimuli and stresses, suggesting that it plays a critical role under these conditions [13]. One of the important functions of ascorbate peroxidase is the protection of cells against photo-oxidative damage through scavenging the hydrogen peroxide and hydroxyl radicals produced [50]. This enzyme is commonly found in photosynthetic cells [51, 52], but reports are accumulating on its occurrence in non-photosynthetic cells like root nodules [53], endosperm [54], tubers [55]. Hydroperoxide lyase breaks down fatty acids producing short-chain aldehydes, which participate in the plant's defense against pathogens and in healing wounds [56].

One protein spot, dihydrokaempferol or dihydroflavonol 4-reductase, that is upregulated during pollen tube development is also known to participate in stress responses, and is involved in flavonoid biosynthesis. Flavonoids protect plants against damage by ultraviolet irradiation and pathogens [57], and play an important role in pollination and sexual reproduction [58].

4.4 Proteins involved in gene regulation

Four protein spots expressed only in pine pollen tubes matched to known proteins involved in varying levels of gene expression (putative nucleic acid binding protein, f-box family protein-like, enhancer-of-zeste, and auxin-induced protein 2). F-box family protein is characterized by a motif that functions as a site of protein-protein interaction [59]. Specifically, this protein has been reported to facilitate transcription elongation by RNA polymerase II [60], inhibit translation [61], facilitate phosphorylation [62], and mediate nuclear entry [63]. Enhancer-of-zeste protein belongs to the Polycomb-group of proteins that have been implicated in multiple examples of gene regulation during development [64]. It was first identified in *Drosophila* as a dominant gain-of-function modifier of the zeste-white interaction, and mutant alleles also produce homeotic transformation. Various homologs have been documented in *Arabidopsis* including *CURLY LEAF* [65], *MEDEA* [66], and *FERTILIZATION-INDEPENDENT ENDOSPERM* [67]. Auxin controls a variety of processes throughout plant development, and at the molecular level it is known to modulate gene expression [68]. Auxin-induced proteins are short-lived nuclear proteins that contain a functional nuclear localization signal sequence and

β - α -fold similar to the β -ribbon DNA recognition motif [69]. These properties suggest a regulatory function of auxin-induced proteins.

Two protein spots that are upregulated in pollen tubes (gag-pol polyprotein and At3g18730) have also been implicated in gene regulation. Retrotransposons typically encode two genes which are transcribed and translated in the cytoplasm of the host as a gag and a gag-pol polyprotein. Gag-pol polyproteins are cleaved by proteases into functional peptides essential for basic replication [70]. At3g18730 (or MGO3) contains tetratricopeptide repeats that are involved in protein-protein interaction [71]. This has been reported to mediate processes such as protein translocation to peroxisomes [72], inhibition of gibberellin sensing [73], control of the cell cycle [74], and regulation of meristem development [75].

4.5 Proteins involved in signal transduction

Four protein spots matched to known proteins involved in signal transduction are expressed only in the pollen tubes (calcium-dependent protein kinase, putative receptor kinase, major surface-like glycoprotein, and remorin-like protein). Calcium-dependent protein kinases are a novel class of Ca^{2+} sensors that are equipped with both kinase and calmodulin-like domains in a single polypeptide [76]. These are involved in various aspects of plant growth and development including pollen tube development [5, 77]. It has been shown that retarding the expression of this gene impairs pollen germination and tube growth [77]. Growing pollen tubes showed higher protein kinase activity in the apical region [5]. Receptor kinases are transmembrane proteins that function to transduce extracellular signals that are involved in processes such as plant growth, development, and defense [78]. Major surface glycoproteins are located on the cell surface and responsible in various cell recognition reactions. Remorin is a plasmodesma-associated protein that is probably involved in cell-to-cell signaling and/or molecular transport [79].

Steroid 22- α -hydroxylase is upregulated in pine pollen tubes. This protein is involved in brassinosteroid biosynthesis, which has been shown to stimulate longitudinal growth of young tissues through cell elongation [80, 81].

4.6 Proteins involved in cell wall formation

The formation of the cell wall is considered to be the major activity of growing pollen tubes [36]. However, this proteomic analysis has only identified one differentially expressed protein related to cell wall formation, *i.e.*, α -expansin. α -Expansins are responsible for the acid-induced loosening of cell walls and typically expressed in rapidly growing cells [82]. On the other hand, since pollen tubes are very active metabolically, sugars are not only utilized as energy source but are also converted to cell wall materials [83]. Therefore, the large number of proteins that are involved in various aspects of cellular metabolism may also be involved in pollen tube wall formation.

4.7 Concluding remarks

This study demonstrates the capacity of proteome analysis in providing new insights into the cellular mechanisms and metabolic pathways underlying pollen tube development in pines. The differentially expressed proteins in pollen tubes represent 6% of the total proteins expressed in this stage of male gametophyte development. Many of these proteins have already been described from pollen tubes of various flowering plants, while others are those that are typically associated with various metabolic activities of plant cells. However, there are also many proteins identified in this study that have not yet been reported from pollen tubes (e.g., phenylcoumaran benzylic ether reductase, kinases, ascorbate peroxidase, f-box family protein, enhancer-of-zeste, gag-pol polypeptide, At3g18730, and many others). Therefore, this study has expanded our knowledge of the proteins that are expressed in the male gametophyte. A deeper analysis of some of these proteins may provide a better understanding of the behavior that characterizes pollen tube development in pines and the mechanisms that regulate this critical stage of sexual reproduction in seed plants.

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