Sex Plant Reprod (2001) 13:259–264

ORIGINAL ARTICLE

Danilo D. Fernando · John N. Owens · Xueshu Yu
Abul K.M. Ekramoddoullah

RNA and protein synthesis during in vitro pollen germination and tube elongation in *Pinus monticola* and other conifers

Received: 30 September 2000 / Revision accepted: 5 February 2001

Abstract Pollen germination and tube elongation in *Pinus monticola* are accompanied by RNA and protein synthesis as shown by the effects of inhibitors such as actinomycin D and cycloheximide, respectively. Pollen grains germinate in the presence of actinomycin D, but further tube elongation is inhibited. This suggests that RNAs needed for germination are already available in the mature ungerminated pollen, but continued tube elongation depends on the synthesis of new RNAs. Using cycloheximide, our results indicate that some proteins essential for germination and tube elongation are not yet available in the mature ungerminated pollen. In *P. monticola*, it appears that these proteins are synthesized at the onset of pollen germination and during tube elongation. The effects of inhibiting transcription and translation in eight other conifers are the same as in *P. monticola*, suggesting a common trend. In *P. monticola*, profiles of pollen grains and pollen tubes varied in the expression of at least ten proteins. Based on the stages examined, the protein profiles of 2-day-old tubes appear to be the most variable with some proteins increasing or decreasing in intensity only at this stage. In *P. monticola*, four proteins (26, 27, 38 and 40 kDa) are differentially expressed during pollen tube development. The most notable is a 26-kDa protein which is specifically expressed in pollen tubes. It is possible that this protein controls a function unique to pollen tubes. This report adds to our knowledge of the regulation of pollen tubes development in conifers. It also offers insights on why development of pollen tubes in conifers takes much longer than in flowering plants.

Keywords Conifers · *Pinus monticola* · Pollen germination · Pollen tube · Protein synthesis

D.D. Fernando (✉)
Department of Environmental and Forest Biology,
State University of New York College of
Environmental Science and Forestry, 350 Illick Hall,
1 Forestry Drive, Syracuse, NY 13210, USA
e-mail: fernando@esf.edu
Fax: +1-315-4706934

J.N. Owens
Centre for Forest Biology, University of Victoria, Victoria,
BC V8W 3N5, Canada

X. Yu · A.K.M. Ekramoddoullah
Natural Resources Canada, Canadian Forest Service,
Pacific Forestry Centre, 506 West Burnside Road, Victoria,
BC V8Z 1M5, Canada

Introduction

One of the remarkable features about the reproductive biology of pines is the prolonged growth of pollen tubes. Pine pollen grains germinate on the nucellus and produce a tube that ceases growth and becomes dormant for several months while not even halfway through the nucellus. Tube elongation resumes in springtime, traverses the rest of the nucellus, and eventually discharges its contents into the egg cytoplasm. From pollination to fertilization, growth of pine pollen tubes spans approximately 15 months (Chamberlain 1935). In spite of this peculiar type of growth, there is very limited information about the regulation of pollen germination and tube elongation in conifers. At the biochemical level, only two studies have been published and these include descriptions of RNA synthesis in generative and tube nuclei in *Pinus ponderosa* (Young and Stanley 1963), and RNA and protein synthesis in germinating *P. taeda* pollen (Frankis 1990).

Our knowledge of the regulation of pollen tube development at the biochemical level is essentially confined to flowering plants. Reports show extreme variation in pollen germination between species if transcription and translation are arrested (Mascarenhas 1971; Franke et al. 1972; Shivanna et al. 1974a, b; Mascarenhas 1975, 1978; Whipple and Mascarenhas 1978; Hoesktra and Bruinsma 1979; Bednarska 1992; Chibi et al. 1994). In addition, these studies have established the effects of inhibitors such as actinomycin D and cycloheximide which control RNA and protein synthesis, respectively. Based on most of the flowering plants studied, Mascarenhas (1993) concluded that the RNAs required for germination and early

tube elongation are already present in the pollen grains at anther dehiscence. The same is true for the proteins that are needed for pollen germination (Shivanna et al. 1974a; Mascarenhas 1993; Chibi et al. 1994). It appears that in flowering plants, synthesis of new proteins is required only for growth of pollen tubes after germination. Many of the proteins present in the mature ungerminated pollen grains are similar to those found in pollen tubes, but their concentration varies from one stage to another (Mascarenhas et al. 1984; Capkova et al. 1987; Frankis 1990). In spite of the appearance of a large number of proteins coincident with pollen germination (Mascarenhas and Mermelstein 1981), only a few proteins have so far been reported to be associated with pollen tube growth (Capkova et al. 1987, 1988, 1994; Tupy et al. 1992; Storchova et al. 1994; Wittink et al. 2000).

Using *P. monticola*, we correlated RNA and protein synthesis with in vitro pollen germination and tube elongation using actinomycin D and cycloheximide, respectively. We also compared the proteins found in mature ungerminated pollen with those of pollen tubes grown for up to 15 days in culture. We believe that new proteins are synthesized in association with pollen tube development. The effects of inhibitors on pollen germination in eight other conifers were also examined.

Materials and methods

Plant material

Pollen cones of *P. monticola* were collected from the Saanich Seed Orchard, Saanichton, British Columbia. Pollen grains of *Cedrus deodara*, *Picea orientalis* and *Pinus aristata* were collected from the University of Victoria, Victoria, British Columbia. Pollen grains of *Abies amabilis*, *Picea sitchensis* and *Pinus contorta* were obtained from Mount Newton Seed Orchard, Saanichton, British Columbia. Pollen grains of *Pinus griffithii* were obtained from the Institute of Forest Genetics, Placerville, California. Pollen grains of *Pinus lambertiana* were obtained from the Dorena Tree Improvement Center, Cottage Grove, Oregon.

Collection of sterile pollen grains

Pollen cones of *P. monticola* were collected 1–3 days before dehiscence and surface sterilized by washing with freshly prepared 70% ethyl alcohol, sterile distilled water, and 1% sodium hypochlorite for 30 s each step. They were then rinsed three times with sterile distilled water for 10 s each step. The pollen cones were blotted dry on sterile paper towels and left in sterile petri dishes for 48–72 h. Petri dish covers were replaced with sterile filter papers and fastened with rubber bands to ensure sterility during drying and shedding of pollen grains. Dried sterile pollen grains were collected and stored in sterile vials at 4°C.

Media composition and culture conditions

Pollen grains were grown on a germination medium containing 100 µg/ml H_3BO_3, 300 µg/ml $Ca(NO_3)_2$, 145 mM sucrose and 0.4% phytagel; the pH was adjusted to 5.8 (Fernando et al. 1998). Cultures were incubated in the dark at 27°C. Actinomycin D and cycloheximide (Sigma Chemicals) were dissolved in 1% dimethylsulfoxide (DMSO). Four concentrations (5, 10, 25 and 50 µg/ml) of each of the inhibitors were prepared. The inhibitors were added to autoclaved media prior to pouring into plates. The controls used in pollen germination and tube elongation experiments contained 1% DMSO.

Initial experiments were done to establish that the DMSO concentration used in this study had no significant effect on pollen germination or tube elongation. The effect of actinomycin D at 60 µg/ml was also tested (data not shown) and the effect on pollen tube growth was similar to the other four concentrations used in this study and to the report of Frankis (1990). Pollen was considered germinated when the length of the pollen tube was equal to or greater than the diameter of the pollen grain. For *P. monticola*, the diameter of the pollen grain is approximately 65 µm.

Protein extraction

Pollen grains and tubes of *P. monticola* were prepared according to Fernando et al. (2000). The protein extraction buffer included 65 mM Tris, 1% sodium dodecyl sulfate, 5% glycerol and 2.5% β-mercaptoethanol at pH 6.8. Samples were collected in a sterile microtube containing 250 µl extraction buffer. 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 30 min. The supernatant was carefully transferred into a new microtube. The protein extract was stored at 4°C until use. Proteins were extracted from pollen tubes at various stages of development, as well as from ungerminated pollen grains taken directly from pollen cones and grown on medium with 10 µg/ml cycloheximide.

Protein quantification

The concentration of proteins was determined according to Ekramoddoullah and Davidson (1995). Briefly, the protein suspension and BSA standard were spotted on a polyvinylidene difluoride membrane (Immobilon-P, Millipore Canada, Toronto). The membrane was stained with 0.1% Coomassie blue R-250 (Bio-Rad) in 50% methanol for 8 min and then destained in 50% methanol containing 10% acetic acid for 8 min at room temperature. The membrane was rinsed with distilled water for 10 min. The stained membrane was scanned using a PDI 420oe scanning densitometer (Protein+dna imageWare systems, Huntington Station, N.Y.) interfaced with a personal computer. Scanning, detection, and quantification were performed according to the instruction manual.

One-dimensional gel electrophoresis

SDS-PAGE was carried out using a Protean II xi System (Bio-Rad) with 1.5 mm thick 12% gel and Laemmli buffer system (Laemmli 1970). A sample volume of 25 µl containing 4 µg protein was applied in each well. Low molecular mass standard markers (14.4–97.4 kDa, Bio-Rad) were used to calibrate the gel. The gels were stained with silver (Hochstrasser et al. 1988).

Analysis of data

Percentage pollen germination was determined by counting the number of pollen grains showing signs of tube emergence and dividing by the total number of pollen grains examined. The number of pollen grains examined was approximately 500 for each treatment/species; each treatment was replicated three times. One-way ANOVA procedures were performed and differences detected with Tukey's honestly significant test using SPSS (SPSS Inc. 1995). This statistical tool was also used to calculate the mean, range and standard error.

Results

Effect of cycloheximide on pollen germination and tube elongation

Pollen grains of *P. monticola* were cultured on media supplemented with various concentrations of cycloheximide (Table 1). After 1-day incubation, a slight indication of pollen tube emergence was evident on medium supplemented with 5 µg/ml cycloheximide. Since the length of pollen tubes was less than the diameter of the pollen grains, these were considered ungerminated. Compared to the control, the length of pollen tubes treated with 5 µg/ml cycloheximide was significantly shorter, while germination on media supplemented with 10, 25 or 50 µg/ml cycloheximide was completely inhibited (Table 1). No change in the length of pollen tubes was observed after 7-day incubation on all media supplemented with cycloheximide. In *P. monticola*, a concentration of 10 µg/ml cycloheximide completely inhibited pollen germination.

To determine whether the effect of cycloheximide was reversible, pollen grains were cultured on medium supplemented with 10 µg/ml cycloheximide for 2, 4 and 6 days, and then transferred onto cycloheximide-free medium (Fig. 1). Our results show that pollen grains previously treated with 10 µg/ml cycloheximide for 2 and 4 days not only germinated when transferred onto cycloheximide-free medium, but the pollen tubes continued to elongate. Pollen grains previously treated with 10 µg/ml cycloheximide for 6 days did not germinate even after 7-day incubation on cycloheximide-free medium. It appeared that pollen grains exposed for up to 4 days to 10 µg/ml cycloheximide were still viable, while exposure for 6 days rendered the pollen grains nonviable.

To examine whether the effect of cycloheximide was specific to a certain stage in pollen tube development, pollen tubes were allowed to elongate on cycloheximide-free medium for 1–15 days (Table 2). Length of pollen tubes was measured before they were transferred onto medium containing 10 µg/ml cycloheximide (Table 2). After 7-day incubation, no difference in tube length was observed in all stages examined. Our results indicate that pollen tube elongation in *P. monticola* was completely arrested by 10 µg/ml cycloheximide irrespective of tube length or the number of days that pollen tubes had been growing in culture.

Table 1 Effects of various concentrations of cycloheximide on pollen germination in *P. monticola*. Mean values followed by different letters indicate significant difference (α=0.05)

Cycloheximide (µg/ml)	Percent Germination Mean (range)	Tube length (µm)	
		One day in culture Mean (SE)	Seven days in culture Mean (SE)
0	90 (80–98)	70a (±5)	365a (±43)
5	0	30b (±10)	30b (±10)
10	0	0	0
25	0	0	0
50	0	0	0

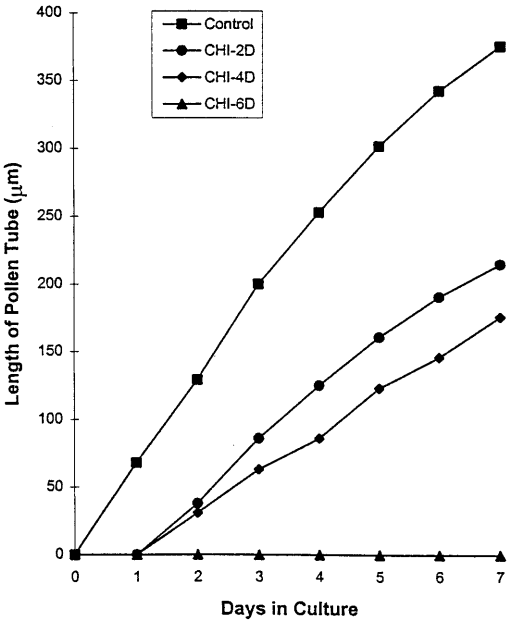

Fig. 1 The effects of growing pollen tubes of *P. monticola* on 10 µg/ml cycloheximide for 2–6 days and then transferring onto cycloheximide-free medium

Table 2 Effect of cycloheximide on pollen tube elongation in *P. monticola* grown initially on cycloheximide-free medium and then transferred onto medium with cycloheximide

Number of days on pollen germination medium	Tube length (µm) after 1-day incubation on cycloheximide-free medium Mean (range)
1	70 (65–88)
2	120 (75–163)
3	190 (113–250)
5	300 (200–375)
10	440 (325–550)
15	600 (463–800)

Effects of actinomycin D on pollen germination and tube elongation

Pollen grains of *P. monticola* were cultured on media supplemented with various concentrations of actinomycin D (Table 3). After 1-day incubation, pollen grains germinated on all the concentrations examined. No significant difference in percentage germination was observed between concentrations, but length of pollen tubes in all media supplemented with actinomycin D was significantly shorter than the control tubes (Table 3). In fact, length of pollen tubes on 5 µg/ml actinomycin D was less than half of that of the control. After 10- and 15-day incubation, elongation of pollen tubes was severely retarded on all media containing actinomycin D.

Table 3 Effects of actinomycin D on pollen germination and tube elongation in *P. monticola*. Mean values followed by the same letter do not differ significantly (α=0.05)

Actinomycin D (µg/ml)	Percent Germination Mean (SE)	Tube length (µm)		
		5 days Mean (SE)	10 days Mean (SE)	15 days Mean (SE)
0	87a (±9.78)	300a (±41)	440a (±45)	600a (±50)
5	85a (±9.80)	141b (±29)	206b (±39)	290b (±40)
10	87a (±8.42)	138b (±35)	155b (±17)	196b (±22)
25	78a (±9.55)	99c (±25)	126c (±19)	165c (±29)
50	80a (±7.05)	86c (±34)	120c (±34)	164c (±42)

Table 4 Effects of cycloheximide and actinomycin D on pollen germination (+ is germinated, – is no germination) in eight conifers belonging to four genera

Species	Cycloheximide (µg/ml)				Actinomycin D (µg/ml)				
	0	10	25	50	0	5	10	25	50
Abies amabilis	+	–	–	–	+	+	+	+	+
Cedrus deodara	+	–	–	–	+	+	+	+	+
Picea orientalis	+	–	–	–	+	+	+	+	+
Picea sitchensis	+	–	–	–	+	+	+	+	+
Pinus aristata	+	–	–	–	+	+	+	+	+
Pinus contorta	+	–	–	–	+	+	+	+	+
Pinus griffithii	+	–	–	–	+	+	+	+	+
Pinus lambertiana	+	–	–	–	+	+	+	+	+

Although significantly shorter than the control, pollen tubes of *P. monticola* grown with actinomycin D at various concentrations continued to elongate slowly throughout the duration of the experiment. It appeared that the effect of actinomycin D was on inhibition of pollen tube elongation, starting very early during pollen tube development. In spite of the more severe inhibition seen after 15 days in culture, tube elongation was never completely arrested (Table 3).

Effects of cycloheximide and actinomycin D on pollen germination in other conifers

Pollen grains of eight other species of conifers belonging to four different genera were cultured on media supplemented with 10, 25 and 50 µg/ml cycloheximide (Table 4). Germination in all species examined was completely blocked in all three concentrations of cycloheximide. These results were similar to the response of *P. monticola* on cycloheximide. Using 5, 10, 25 and 50 µg/ml actinomycin D, germination also occurred in all the species examined; this is similar to the effect exhibited by *P. monticola* (Table 4). It appeared that the effects of cycloheximide and actinomycin D were not specific to *P. monticola*, but rather common to the conifers examined.

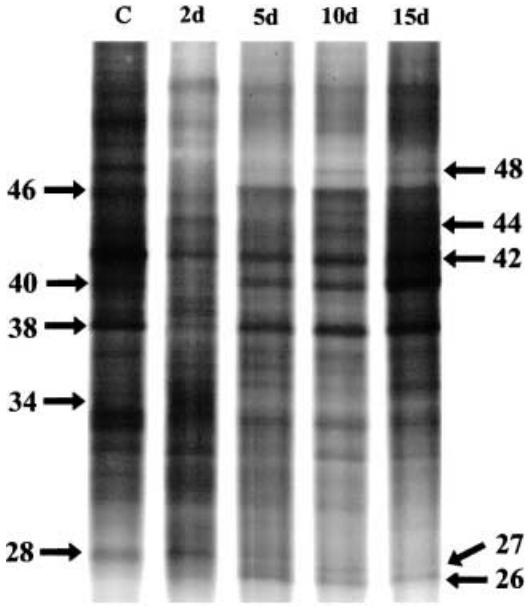

Fig. 2 Protein profiles of *Pinus monticola* pollen tubes at 2, 5, 10 and 15 days after one-dimensional gel electrophoresis. The molecular weights are in kDa. *c* Control (ungerminated pollen)

Protein profiles of ungerminated pollen and pollen tubes

Although there is general similarity in the protein profiles of *P. monticola* pollen grains and tubes grown for up to 15 days in culture, a few of the proteins showed expression unique to pollen tube development (Fig. 2). The control lane represents the proteins from ungerminated pollen grains taken directly from pollen cones. No difference in protein profiles was observed when compared with ungerminated pollen grains grown on medium with 10 µg/ml cycloheximide (data not shown). At least ten of the proteins showed differences and the most notable was a 26-kDa protein that was expressed in all pollen tubes examined, but not in mature ungerminated pollen. Another protein, which was preferentially expressed in the pollen tube, was a 27-kDa protein; it was found only starting in 5-day old tubes and then it became faint in 15-day old tubes. A 28-kDa protein was found in ungerminated pollen and 2-day-old tubes but its intensity gradually declined until it was no longer detected in 15-day-old tubes. Of the proteins common to all the stages examined, two proteins (34 and 44 kDa) appeared most intense in 2-day-old tubes and four other proteins (38, 40, 46 and 48 kDa) were distinctly less intense in 2-day-old tubes. Two proteins (38 and 40 kDa) became most intense in 15-day-old pollen tubes. A 42 kDa protein appeared intense in ungerminated pollen, but became drastically less intense as the tubes developed.

Discussion

Pollen grains of *P. monticola* and the other eight conifers examined in this study are completely inhibited from

germinating when protein synthesis is blocked. Blocking protein synthesis also completely inhibits pollen tube elongation in germinated pollen grains of *P. monticola*. These results suggest that the mature ungerminated pollen grains of conifers do not yet contain the proteins that are required to initiate germination, and the proteins synthesized during germination are not sufficient to sustain continuous tube elongation. It appears that de novo protein synthesis is actively occurring during pollen germination and tube elongation, and is essential for continued tube growth. Our results are supported by the work of Frankis (1990) who showed that in *P. taeda*, proteins are synthesized at different times during germination and early tube growth. The timing of protein synthesis in conifers differs from that reported from flowering plants where the mature ungerminated pollen grains already contain all the proteins required for germination and early tube elongation (Mascarenhas 1993). In this regard, it appears that conifer pollen grains and tubes behave differently from those of flowering plants. This implies that once conifer pollen grains reach the nucellus, they still have to produce new proteins before germination can proceed. Furthermore, synthesis of new proteins has to occur continuously throughout the process of pollen tube elongation. Our results contribute to our understanding about why pollen tube development in conifers lags behind that of their phylogenetically advanced relatives, the flowering plants.

Pollen germination in *P. monticola* and the eight other conifers examined is not arrested when transcription is blocked. In *P. monticola*, the effect of blocking transcription is manifested by the slowing down of pollen tube elongation. Frankis (1990) showed that in *P. taeda*, blocking RNA synthesis with actinomycin D inhibits tube elongation after as early as 12 h, and the inhibition becomes more prominent after 2 days. We have grown pollen tubes of *P. monticola* with actinomycin D for up to 15 days and our results support the report of Frankis (1990). It appears that the effect of blocking transcription during germination is expressed immediately and maintained for at least 15 days. This means that the RNAs available in mature ungerminated pollen are not sufficient to sustain the normal rate of tube elongation, even at an early stage of pollen tube development. Blocking transcription in conifers appears to have the same effect as in most flowering plants (Mascarenhas 1993).

It appears that conifer pollen grains already contain the RNAs necessary for pollen germination. However, it is important that synthesis of new RNAs occurs continuously during and after pollen germination; otherwise pollen tubes are inhibited from elongating at a rate similar to the control. In *P. taeda*, Frankis (1990) attributed the severely retarded pollen tube growth in the presence of actinomycin D to the lack of RNAs essential for normal tube growth. In pines, the generative and tube nuclei have been shown to be active in synthesizing RNAs at a very early stage of pollen germination and the RNAs synthesized have been identified as rRNAs (Young and Stanley 1963; Frankis 1990).

In *P. monticola*, many of the proteins present in the mature ungerminated pollen grains are similar to those found in pollen tubes. Our results support the general observation that there is a close similarity between different stages of pollen tube development (Mascarenhas et al. 1984; Capkova et al. 1987; Frankis 1990). Mascarenhas (1993) suggested that a large number of genes expressed in pollen grains have functions in tube growth. In *P. monticola*, variation in the expression of genes at the protein level is evident in at least ten of the polypeptides. The 28-kDa protein which is found in ungerminated pollen and 2-day-old tubes but disappears in later stages is probably essential only during germination and early tube growth. The appearance of a 27-kDa protein in pollen tubes is coincident with the occurrence of pollen tube branches. It has been observed that pine pollen tubes branch after being in culture for 5 days (Fernando et al. 1997).

The major biochemical processes that occur during pollen tube development include formation of the components of internal and plasma membranes, and the pollen tube wall. It is possible that some of the proteins synthesized during pollen germination and tube elongation in *P. monticola* are utilized in these processes. In *Nicotiana tabacum*, Capkova et al. (1987) reported that a 65-kDa protein specifically expressed during pollen tube growth plays an important role in pollen tube wall formation. A 69-kDa glycoprotein associated with the vegetative membranes and cell wall has been shown to increase in concentration during pollen tube growth (Wittink et al. 2000). If there is a protein in *P. monticola* that can be correlated with tube wall formation, it might be either of the two proteins (38 and 40 kDa) that increase proportionately with time in culture or predominate in later stages. Since these two proteins decrease in concentration in 2-day-old tubes, it is possible that they are utilized during germination and then synthesized again during pollen tube development. At least four proteins (26, 27, 38 and 40 kDa) are differentially expressed during pollen tube development and are probably essential for pollen tube growth.

Based on all the stages examined, the protein profiles of 2-day-old tubes appear to be the most variable in *P. monticola*. The proteins that are less intense in 2-day-old tubes are probably used up during germination and early tube elongation, while those that are more intense are probably needed during pollen tube growth. Two proteins intensify in 2-day-old tubes, but the most intense is the 34 kDa protein. This protein might be similar to the 36-kDa protein that predominates in 2-day-old tubes in *P. taeda* as described by Frankis (1990).

So far, no protein unique to pollen tubes has been described in any conifer. In this paper, we report a 26-kDa protein that is specifically expressed in pollen tubes at several stages during culture, but not in the mature ungerminated pollen. The preferential synthesis of this protein and its accumulation in pollen tubes suggest that it may be important for pollen tube growth in *P. monticola*. Further study is needed to establish the unique occur-

rence of this protein in pine pollen tubes. In flowering plants, a few proteins have been described to be specifically expressed in pollen tubes, and these include the 66 and 69 kDa proteins in *Nicotiana tabacum* (Capkova et al. 1987, 1988, 1994; Tupy et al. 1992; Storchova et al. 1994). Recently, Wittink et al. (2000) reported that the 69-kDa protein appears to be identical to the glycoprotein NTP303 which is expressed in pollen grains of *N. tabacum*.

We have described some of the proteins present in *P. monticola* during in vitro pollen tube development. These proteins might be important in the regulation of pollen germination and tube elongation, but there is no information as to the specific identity and function of any of these proteins. In spite of the amount of work in flowering plants, no gene uniquely associated with pollen germination or tube growth has yet been identified (Mascarenhas 1993; Taylor and Hepler 1997). According to Mascarenhas (1993), the successful isolation of genes expressed specifically in the pollen tube would provide conclusive evidence of a unique genetic program during pollen tube development. Since many of the mRNAs might already be present in mature conifer pollen grains, a cDNA library coupled with differential screening would be the logical approach to isolate the genes involved in this stage of male gametophyte development.

Acknowledgements This research was supported by a grant (A-1982) from the Natural Sciences and Engineering Research Council of Canada to J.N.O. We thank David R. Johnson for providing pollen grains of *P. griffithii*, Luke Chandler for collecting pollen grains of *Abies amabilis* and *Picea sitchensis*, Ben Forward for helping in protein extraction and Doug Taylor for technical support.

References

Bednarska E (1992) The localization of nonspecific esterase and cholinesterase activity in germinating pollen and in pollen tube of *Vicia faba* L. The effect of actinomycin D and cycloheximide. Biol Plant 34: 229–240

Căpková V, Hrabetova E, Tupy J (1987) Protein changes in tobacco pollen culture: a newly synthesized protein related to pollen tube growth. J Plant Physiol 130: 307–314

Căpková V, Hrabetova E, Tupy J (1988) Protein synthesis in pollen tubes: preferential formation of new species independent of transcription. Sex Plant Reprod 1:150–155

Căpková V, Zbrožek J, Tupy J (1994) Protein synthesis in tobacco pollen tubes: preferential synthesis of cell-wall 69-kDa and 66-kDa glycoproteins. Sex Plant Reprod 7:57–66

Chamberlain CJ (1935) Gymnosperms. Dover, New York

Chibi F, Matilla AJ, Angosto T, Garrido D (1994) Changes in polyamine synthesis during anther development and pollen germination in tobacco (*Nicotiana tabacum*). Physiol Plant 92:61–68

Ekramoddoullah AKM, Davidson JJ (1995) A method for the determination of conifer foliage protein extracted using sodium dodecyl sulfate (SDS) and mercaptoethanol. Phytochem Analysis 6:20–24

Fernando DD, Owens JN, Aderkas P von (1998) In vitro fertilization from co-cultured pollen tubes and female gametophytes of Douglas fir (*Pseudotsuga menziesii*). Theor Appl Genet 96:1057–1063

Fernando DD, Owens JN, Aderkas P von, Takaso T (1997) In vitro pollen tube growth and penetration of female gametophyte in Douglas fir (*Pseudotsuga menziesii*). Sex Plant Reprod 10:209–216

Fernando DD, Owens JN, Misra S (2000) Transient gene expression in pine pollen tubes following particle bombardment. Plant Cell Rep 19:224–228

Franke WW, Herth W, Van der Woude WJ, Moore DJ (1972) Tubular filamentous structures in pollen tubes: possible involvement as guide elements in protoplasmic streaming and vectorial migration of secretory vesicles. Planta 105:317–341

Frankis RC Jr (1990) RNA and protein synthesis in germinating pine pollen. J Exp Bot 41:1469–1473

Hochstrasser DF, Harrington MG, Hochstrasser AC, Miller MJ, Merril CR (1988) Methods for increasing the resolution of two-dimensional protein electrophoresis. Ann Biochem 173: 424–435

Hoekstra FA, Bruinsma J (1979) Protein synthesis of binucleate and trinucleate pollen and ist relationship to tube emergence and growth. Planta 146:559–566

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227:680–685

Mascarenhas JP (1993) Molecular mechanisms of pollen tube growth and differentiation. Plant Cell 5:1303–1314

Mascarenhas JP (1971) RNA and protein synthesis during pollen development and tube growth. In: Heslop-Harrison J (ed) Pollen:development and physiology. Butterworth, London

Mascarenhas JP (1975) The biochemistry of angiosperm pollen development. Bot Rev 41:259–314

Mascarenhas JP (1978) Ribonucleic acids and proteins in pollen germination. IV International Palynological Conference, Lucknow 1:400–406

Mascarenhas JP, Mermelstein J (1981) Messenger mRNAs: their utilization and degradation during pollen germination and tube growth. Acta Soc Bot Pol 50:13–20

Mascarenhas NT, Bashe D, Eisenberg A, Willing RP, Xiao CM, Mascarenhas JP (1984) Messenger mRNAs in corn pollen and protein synthesis during germination and pollen tube growth Theor Appl Genet 68:323–326

Shivanna KR, Jaiswal VS, Mohan Ram HY (1974a) Inhibition of gamete formation by cycloheximide in pollen tubes of *Impatiens balsamina*. Planta 117:173–177

Shivanna KR, Jaiswal VS, Mohan Ram HY (1974b) Effect of cycloheximide on cultured pollen grains of *Trigonella foenum-graecum*. Plant Sci Lett 3:335–339

SPSS, Inc (1995) SPSS Statistical Algorithms. Chicago, Ill

Storchova H, Capkova V (1994) A *Nicotiana tabacum* mRNA encoding a 69-kDa glycoproteins occurring abundantly in pollen tubes is transcribed but not translated during pollen development in the anthers. Planta 192:441–445

Taylor LP, Hepler PK (1997) Pollen germination and tube growth. Annu Rev Plant Physiol 48:641–491

Tupy J, Rihova L, Capkova V, Zarsky V (1992) Differentiation and maturation of tobacco pollen in situ and in suspension culture. In: Ottaviano E, Mulcahy DL, Sari Gorla M, Bergamini Mulcahy G (eds) Angiosperm pollen and ovules. Springer, New York Berlin Heidelberg

Whipple AP, Mascarenhas JP (1978) Lipid synthesis in germinating *Tradescantia* pollen. Phytochemistry 17:1273–1274

Wittink FRA, Knuiman B, Derksen J, Čapková V, Twell D, Schrauwen JAM, Wullems GJ (2000) The pollen-specific gene *Ntp303* encodes a 69-kDa glycoprotein associated with the vegetative membranes and the cell wall. Sex Plant Reprod 12:276–284

Young LTC, Stanley RG (1963) Incorporation of tritiated nucleosides thymidine, uridine and cystidine in nuclei of germinating pine pollen. Nucleus 6:83–90