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ORIGINAL ARTICLE

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Expression of β-galactosidase and β-xylosidase genes during microspore and pollen development

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Abstract Tobacco (*Nicotiana tabacum* L.) microspores at the time of mitosis are characterized by the abundant occurrence of 92- and 98-kDa glycoproteins (GP92 and GP98). GP92 is a soluble protein while GP98 is bound to the insoluble microspore fraction. Both glycoproteins were isolated by affinity chromatography and SDS–PAGE and analysed by MS. Peptide sequences were determined by μ-HPLC/nano-ESI–MS/MS (electrospray ionization tandem MS). GP92 displayed homology to β-galactosidase (EC 3.2.1.23) and GP98 to β-xylosidase (EC 3.2.1.37) from *Arabidopsis thaliana* (L.) Heynh. The activities of the two enzymes in microspore and pollen extracts of tobacco exhibited similar developmental changes to the occurrence of GP92 and GP98, with a maximum around microspore mitosis. These two glycoproteins are the first identified enzymes characteristic of mitotic microspores. *Arabidopsis* transcriptomic data for five β-galactosidase and three β-xylosidase genes abundantly expressed in pollen were verified by reverse transcription–PCR of RNA from different stages of *Arabidopsis* pollen development and from various parts of the sporophyte. The results showed abundant expression of two genes (At5g20710, At1g31740) homologous to tobacco GP92 in microspores and early pollen, and of three genes (At5g56870, At2g16730 and At4g35010) in maturing pollen. Analysis of β-xylosidases showed abundant expression of a late pollen-specific gene At3g62710 and low expression of an early gene At5g10560. It is suggested that the early β-galactosidase and β-xylosidase genes may participate in cell wall loosening associated with pollen expansion after microspore mitosis and that the products of the late genes may play a role in cell expansion during pollen germination.

Keywords *Arabidopsis* · Glycoprotein · Glycosyl hydrolase · *Nicotiana* · Pollen development · Transcriptome

Abbreviations ConA: Concanavalin A · ESI: Electrospray ionization · GP: Glycoprotein · IEF: Isoelectric focusing · MALDI–TOF: Matrix-assisted laser desorption/ionization time-of-flight · MS/MS: Tandem mass spectrometry · RT–PCR: Reverse transcription–polymerase chain reaction

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Introduction

Genes expressed during male gametophyte development are generally classified as early and late (McCormick 1993). Transcripts of early genes are first detected soon after meiosis and are undetectable in mature pollen. Transcripts of the late genes are first detected after microspore mitosis and continue to accumulate as pollen matures. Genes with less-distinct temporal expression patterns have also been characterized (Twell et al. 1993). Analysis of the pollen transcriptome from *Arabidopsis thaliana* revealed 992 and 1,584 genes in mature dormant and hydrated pollen grains, respectively (Becker et al. 2003; Honys and Twell 2003). Depending on the sporophytic datasets used, the protocols for purification of pollen grains and the data analysis performed, the estimated proportion of putative male gametophyte-specific genes falls within the range 10–39% (Becker et al. 2003; Honys and Twell 2003); however, the function of the vast majority remains unknown.

A number of late pollen-specific genes have been identified and characterized (reviewed in Twell 2002). Early genes have been identified after differential screening of cDNA libraries from whole anthers (Roberts et al. 1991; Shen and Hsu 1992; Varbanova et al. 2003). Analysis of cDNA from isolated microspores of *Brassica napus* showed an accumulation of Bp4 gene transcript during microspore development. Its expression was highest at microspore mitosis and absent during the late stages of pollen maturation (Albani et al. 1990). Until now, the only microspore-specific gene that has been identified is NTM19 from *Nicotiana tabacum*, which encodes a 10.8-kDa protein of unknown function (Oldenhof et al. 1996). NTM19 transcripts appear after the tetrad stage and disappear at microspore mitosis.

Previous studies on gene expression by protein analysis have shown that phases of microsporogenesis and gametophytic pollen development in tobacco are characterized by the occurrence of cell-specific glycoproteins (Říhová et al. 1996; Hrubá and Tupý 1999). Most notable are a cytosolic 92-kDa glycoprotein (GP92) and an insoluble 98-kDa glycoprotein (GP98), which are abundantly expressed at microspore mitosis and disappear during the subsequent pollen development. Both glycoproteins are characterized by N-linked high-mannose- and/or hybrid-type glycans.

Mass-spectrometric analysis of these glycoproteins, presented here, revealed high homology of GP92 to glycosyl hydrolase family 35 (β-galactosidase) and of GP98 to glycosyl hydrolase family 3 (β-xylosidase). The regulation of expression of genes for β-galactosidase and β-xylosidase during microspore and pollen development was further studied using *Arabidopsis* pollen transcriptomic data verified by reverse transcription–polymerase chain reaction (RT–PCR) analysis.

Materials and methods

Plant material

Tobacco (*Nicotiana tabacum* L. cv. Samsun) plants used for protein analysis of the male gametophyte were grown in a greenhouse from April to October under daylight. Microspores and pollen were isolated from the anthers at the following developmental stages: early (−2), mid- (−1), late (0) and mitotic (1) microspores, and early (2), mid- (3, 4), and maturing (5) pollen, and almost mature pollen 1 day before shedding (6), as described previously (Hrubá a Tupý 1999).

For RNA isolation and RT–PCR analysis, *Arabidopsis thaliana* (L.) Heynh. ecotype Landsberg *erecta* was used. Microspores, pollen, leaves and stems were harvested from plants grown in controlled-environment cabinets at 21°C under illumination of 100 μmol photons m^{-2} s^{-1} with a 16-h photoperiod. Roots were taken from plants grown in the dark at 22°C in 0.5× Murashige and Skoog medium (Sigma) supplemented with 1% (w/v) sucrose under constant shaking for 6 weeks. Pollen for microarray experiments was harvested from two independently grown plant populations at developmental stage 5.9 (Boyes et al. 2001), according to Honys and Twell (2003). Isolated spores from three stages of immature male gametophytes were obtained by modification of the protocol of Kyo and Harada (1985, 1986). After removal of open flowers, inflorescences (bud clusters) from 400 plants were collected and gently ground using a mortar and pestle in 0.3 M mannitol. The slurry was filtered through 100-μm and 53-μm nylon mesh. Mixed spores were concentrated by centrifugation (50-ml Falcon tubes; 450 *g*, 3 min, 4°C). Concentrated spores were loaded onto the top of a 25%/45%/80% Percoll step gradient in a 10-ml centrifuge tube and centrifuged (450 *g*, 5 min, 4°C). Three fractions were obtained containing: microspores mixed with tetrads (1), microspores mixed with bicellular pollen (2) and tricellular pollen (3). Fraction 2 was diluted with 1 vol. of 0.3 M mannitol loaded onto the top of a 25%/30%/45% Percoll step gradient and centrifuged again under the same conditions. Three sub-fractions of immature pollen were obtained containing: microspores (2.1), a mixture of microspores and bicellular pollen (2.2), and bicellular pollen (2.3). Spores in each fraction were concentrated by centrifugation (Eppendorf tubes; 2,000 *g*, 1 min, 4°C) and stored at −80°C. The purity of isolated fractions was determined by light microscopy and staining with 4′,6-diamidino-2-phenylindole (DAPI) according to Park et al. (1998). Vital staining of isolated spore populations was assessed by treatment with fluorescein 3′,6′-diacetate (FDA; Eady et al. 1985).

Protein extraction and two-dimensional (2D) SDS–PAGE

Microspores and pollen were isolated from 20 flower buds and homogenized in 600 μl of cold 20 mM Tris–HCl (pH 8.0) in a Mini-Beadbeater 3110BX (BioSpec) using glass beads 1.0 mm in diameter. Cell disruption was achieved by beating at 4,800 rpm for 2 min, interrupted by short cooling on ice after 1 min. The homogenate was transferred to an Eppendorf tube using a micropipette and centrifuged at 20,000 *g*, 6°C for 15 min. The supernatant represented the soluble fraction. The pellet was resuspended in the homogenization buffer supplemented with 20 mM $CaCl_2$ and 0.3% (w/v) SDS and re-centrifuged under the same conditions. The supernatant represented the insoluble protein fraction. Nucleic acids present in the extracts were digested with 2.5 μg ml^{-1} of ribonuclease A from bovine pancreas (type XII-A; Sigma) in 200 mM Tris–HCl (pH 8.0) containing 25 mM NaCl, 2 mM $MgCl_2$ for 1 h at 4°C. Proteins were then precipitated with 5 vol. acetone overnight at −20°C and sedimented at 20,000 *g* for 15 min. The pellet was dissolved in sample buffer [7 M urea, 2 M thiourea, 2 mM tributylphosphine, 4% (w/v) Chaps, 0.2% (v/v) ampholytes 3/10] for isoelectric focussing (IEF).

The samples were loaded on an 11-cm IPGready strip (pH 3–10) and IEF was run using an IEF Protean Cell (BioRad) according to the manufacturer's instructions. In the second dimension the proteins were separated on a 10% polyacrylamide gel with SDS according to standard protocols (Laemmli 1970).

Protein extraction and one-dimensional (1D) SDS–PAGE

Proteins for 1D SDS–PAGE were extracted in the same way as for 2D SDS–PAGE except for the composition of extraction buffer. Soluble proteins were extracted with 50 mM Tris–HCl (pH 6.8), 10% (w/v) sucrose, 1% (v/v) mercaptoethanol, 0.02% (w/v) sodium azide, and for extraction of insoluble proteins this buffer was supplemented with 1% (w/v) SDS. Proteins were precipitated with 5 vol. acetone overnight at −20°C and separated by1D SDS–PAGE as described previously (Hrubá and Tupý 1999).

Protein staining

Proteins were visualized by Coomassie brilliant blue R-250 (CBB) or electrophoretically transferred from the gel to nitrocellulose (NC membrane, 0.45 μm; Serva) by electroblotting in 20 mM Tris–HCl, 150 mM glycine and 20% (v/v) methanol at 200 mA for 2 h. Glycoproteins on Western blots were detected with concanavalin A (ConA) and peroxidase staining, and the bound peroxidase was visualized with 4-chloro-naphthol (Towbin et al. 1979).

Isolation of GP92 and GP98 for MS analysis

Soluble and insoluble protein fractions were extracted from microspores at a developmental stage around mitosis in the same way as for 2D and 1D SDS–PAGE. The soluble fraction containing GP92 and the insoluble fraction with GP98 were obtained from microspores of 360 and 120 flower buds, respectively. The minor amount of GP98 present in the soluble fraction was removed by ultracentrifugation at 60,000 *g* at 6°C for 2 h 15 min. Glycoproteins were separated from non-glycosylated proteins by affinity chromatography on ConA-agarose as described previously (Hrubá and Tupý 1999). GP92 was separated by IEF–SDS–PAGE and GP98 by 1D SDS–PAGE as described above. GP92 and GP98 were stained with CBB, cut from the gels and stored in 5% (v/v) acetic acid for MS analysis.

Proteolytic digestion and sample preparation

The CBB-stained glycoprotein spot cut from the gel, was chopped into small cubes (approx. 1 mm^3) and washed several times with 10 mM dithiothreitol, 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% (v/v) acetonitrile. After complete de-staining, the gel was washed with water, shrunk by dehydration with acetonitrile and re-swollen in water. Next, the gel was partly dried using a SpeedVac concentrator and reconstituted with cleavage buffer containing 0.01% (v/v) 2-mercaptoethanol, 0.1 M 4-ethylmorpholine acetate, 10% (v/v) acetonitrile, 1 mM $CaCl_2$ and sequencing-grade trypsin (Promega; 50 ng μl^{-1}). Digestion was carried out overnight at 37°C. The resulting peptides were extracted with acetonitrile/trifluoroacetic acid (40:1, v/v) and subjected to mass spectrometric analysis.

Mass-spectrometric analysis

Mass spectra were measured on a matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI–TOF) mass spectrometer (BIFLEX; Bruker-Franzen, Bremen, Germany) equipped with a nitrogen laser (337 nm) and gridless delayed extraction ion source. Ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of the peptide standard somatostatin (Sigma). A saturated solution of α-cyano-4-hydroxy-cinnamic acid in acetonitrile/trifluoroacetic acid (50:0.2, v/v) was used as a MALDI matrix. A 1-μl aliquot of matrix solution was mixed with 1 μl of the sample on the target and the droplet was allowed to dry at ambient temperature. MALDI–MS spectra were interpreted with the program ProFound (http://129.85.19.192/profound_bin/webProFound.exe).

A 2-μl aliquot of the tryptic peptide mixture was applied to a capillary column (150 mm long, 0.1 mm i.d.) packed with 10 cm of C18 RP resin (MAGIC AQ; MichromBioresources, USA). Peptides were separated using gradient elution: 65 min from acetonitrile/acetic acid (5:0.5, v/v) to acetonitrile/acetic acid (40:0.4, v/v) and 15 min from acetonitrile/acetic acid (40:0.5, v/v) to acetonitrile/acetic acid (70:0.4, v/v) at flow rate of 1 μl min^{-1}. The column was connected to an LCQ^{DECA} ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA) equipped with a nanoelectrospray ion source.

Spray voltage was held at 1.2 kV, and tube lens voltage was 30 V. The heated capillary was kept at 175°C with a voltage of 10 V. Collision energy was kept at 42 units and the activation time was 30 ms. Positive-ion full scans were acquired over m/z range 350–1,300. Collisions were done from the top three most intense ions in each full MS scan. Dynamic exclusion was enabled with a repeat count of 2. Selected collisional spectra were interpreted manually and derived sequences were searched against the *Arabidopsis thaliana* database using the MS-Pattern program at the Protein prospector v4.0.5 web site (http://prospector.ucsf.edu/ucsfhtml4.0/mspattern.htm) and nr database using the MS-BLAST program (http://dove.embl-heidelberg.de/Blast2/msblast.html).

A phylogenetic tree was constructed after multiple sequence alignments using the CLUSTALW program (http://clustalw.genome.jp/sit-bin/nph-clustalw). The number of N-glycosylation sites was determined manually from amino acid sequences of particular proteins.

Assay of β-galactosidase and β-xylosidase

Microspores and pollen isolated from 20 flower buds were ground in a frozen glass mortar in 50 mM Mes (pH 6.0) containing 500 mM NaCl, and then centrifuged at 20,000 *g* for 15 min at 4°C. The activities of galactosidase and xylosidase were assayed in the supernatant according to Cleemput et al. (1997). The assay mixture consisted of 300 µl of 50 mM Mes (pH 6.0), 100 µl substrate (10 mM *p*-nitrophenyl-β-D-galactopyranoside or 10 mM *p*-nitrophenyl-β-D-xylopyranoside dissolved in 50 mM Mes, pH 6.0) and 300 µl of microspore or pollen extract. After incubation at 37°C for 2 h, the reaction was stopped by adding 300 µl of 1 M Na_2CO_3. In control samples the reaction was stopped immediately after the addition of cell extracts. The release of *p*-nitrophenol from the substrates was determined colorimetrically at 405 nm. Calibration curves were obtained using 0–0.5 mM *p*-nitrophenol.

RNA extraction and RT–PCR

RNA extraction and RT–PCR were done as described previously (Lalanne et al. 2004). The same starting amount of RNA was used for the RT reaction and 1 µl of 50-fold-diluted RT product was used for PCR. Therefore, the RT–PCR data could be considered semiquantitative. The following gene-specific forward and reverse primers were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi):

- At5g20710-F, 5′-ACTTCTCAGCGTTTCCGTTG-3′;
- At5g20710-R, 5′-CCCGAGACCATTTAAGTCCA-3′;
- At1g31740-F, 5′-CAGAATCACCTTCGACATGG-3′;
- At1g31740-R, 5′-TTGACAAGTGAGGGGTTTCC-3′;
- At4g35010-F, 5′-GCTTGGTGTTCTCACAGGA-3′;
- At4g35010-R, 5′-TATCTTCCAACGCCTTCTCC-3′;
- At2g16730-F, 5′-TCTTAGGTTTGGGCTCTGGA-3′;
- At2g16730-R, 5′-ATCAAACCTTTCCCCAATCC-3′;
- At5g56870-F, 5′-TGAGTGTTGCAGTGGGTCTC-3′;
- At5g56870-R, 5′-GTCCATCTCACACCGGAACT-3′;
- At3g62710-F, 5′-GCGGTTCATGGCCACAATACTT-3′;
- At3g62710-R, 5′-CCTCCGTCCCCAACAAAATGT-3′;
- At3g10560-F, 5′-GCTCAATCGCAGATTGTGTGTG-3′;
- At3g10560-R, 5′-TCCTGGTGAGGATCCGAAGGTA-3′;
- At3g47000-F, 5′-GCCACTGTCTTCCCTCACAACA-3′;
- At3g47000-R, 5′- GAAGCGATGGTGTTCCCTTCAT-3′.

Where possible, primers were designed to span an intron.

Transcriptomic data analysis

DNA chip hybridization Total RNA was extracted from 50 mg of isolated spores at each developmental stage using the RNeasy Plant Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The yield and purity of RNA were determined spectrophotometrically using an Agilent 2100 Bioanalyser (Agilent Technologies, Boblingen, Germany) at NASC.

Biotinylated target RNA was prepared from 20 µg of total RNA as described in the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA). Double-stranded cDNA was synthesized using SuperScript Choice System (Life Technologies) with oligo$(dT)_{24}$ primer fused to T7 RNA polymerase promoter. Biotin-labeled target complementary RNA (cRNA) was prepared by cDNA in vitro transcription using the BioArray High-Yield RNA Transcript Labeling Kit (Enzo Biochem, New York, NY, USA) in the presence of biotinylated UTP and CTP.

Arabidopsis ATH1 Genome Arrays containing over 24,000 genes were hybridized with 15 µg labelled target cRNA for 16 h at 45°C. Microarrays were stained with streptavidin–phycoerythrin solution and scanned with an Agilent 2500A GeneArray Scanner.

Data analysis Male gametophytic transcriptomic datasets (Honys and Twell 2003) are publicly available at the GARNet website (http://www.arabidopsis.info). Samples were labeled as follows: UNM, uninucleate microspore; BCP, bicellular pollen; TCP, tricellular pollen; MPG, mature pollen. Sporophytic data from public baseline GeneChip experiments used for comparison with the pollen transcriptome were downloaded from the GARNet website (http://www.arabidopsis.info; Craigon et al. 2004). Dataset codes downloaded from the GARNet website were as follows: INF—inflorescence (Turner—Turner_A-5-Turne-WT-Top1–2_SLD); COT—seedlings at cotyledon stage 1.0 [Cornah (COT1–3), Villadsen (COT2–1), Rente (COT4–1), Greville (COT5–3)-Cornah_A4-cornah-wsx_SLD_REP1–3, Villadsen_A-1-villa-zer_SLD, Short_A2-mcain-con, A3-Rente-WS2-Control_SLD, Greville_A-01-grevi-CC1–3_SLD]; LEF—leaves [Heggie (LEF1–2), Lloyd (LEF2–3), Greco (LEF3–1)-A5-HEGGI-CAW, A4-LLOYD-CON_REP1–3, A2-Greco-WT]; PET—petioles (Millenaar—Millenaar_A1-MILL-AIR-REP1–3); STM—stem base (Turner—Turner_A-7-Turne-WT-Base1–2_SLD); ROT—roots [Yap (ROT1–1), Urwin (ROT2–1), Filleur (ROT3–2)—Yap_A2-AMF, Urwin_A-1-Urwin-Con_SLD, Sophie_A1-fille-WTw_SLD]; RHR—root hair zone (Jones—Jones_A1-jones-WT1–2_SLD); SUS—cell suspension culture [Willats (SUS1–3), Swid-

zinski (SUS2–3)—A1-WILLA-CON-REP1–3, Swidzinski Control AGA Replicate 1–3]. The number after the dash indicates the number of replicates used in each experiment. All gametophytic and sporophytic datasets were normalized using freely available dChip 1.3 software (http://www.dchip.org). The reliability and reproducibility of analyses were ensured by (i) the use of duplicates or triplicates in each experiment, (ii) the normalization of all 26 arrays to the median probe intensity level and (iii) the use of normalized CEL intensities of all arrays for the calculation of model-based gene expression values based on the Perfect Match-only model (Li and Wong 2001a, 2001b). A particular gene was scored as "expressed" when it gave a reliable expression signal in all replicates. Expression signal value "0" means that the detection call value was not "present" in all replicates provided.

Results

Occurrence of GP92 and GP98 during microspore and pollen development in tobacco

The ConA-binding pattern of glycoproteins on Western blots confirmed previous results (Hrubá and Tupý 1999) that GP92 occurs abundantly in the soluble fraction of mitotic microspores (Fig. 1a). GP98 present in this fraction can be removed by ultracentrifugation (Hrubá and Tupý 1999). It occurs in dividing microspores as the dominant insoluble glycoprotein according to detection with ConA (Fig. 1b). Developmental changes in patterns of ConA-binding insoluble glycoproteins show that GP98 is mostly expressed in late and mitotic microspores and in early pollen (Fig. 2).

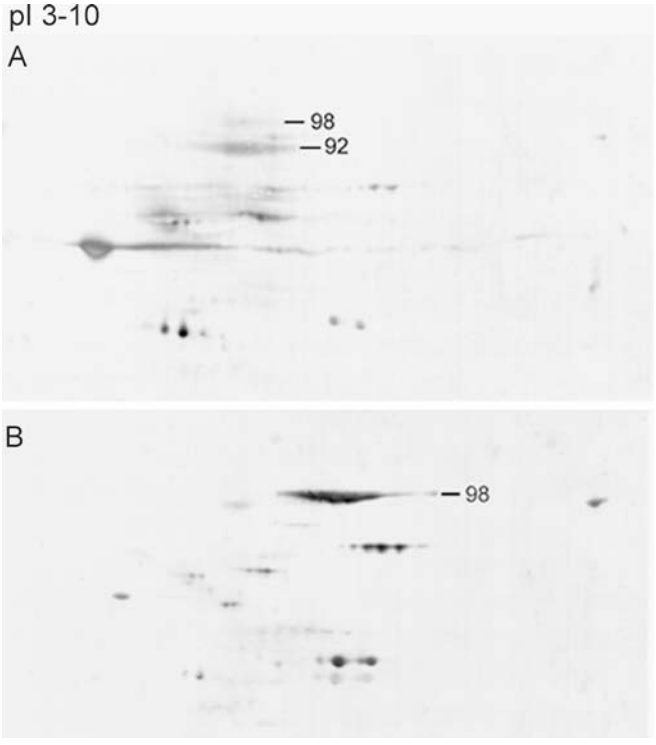

Fig. 1a,b Con-A affinoblots from IEF–SDS–PAGE of soluble (**a**) and insoluble (**b**) glycoproteins extracted from tobacco (*Nicotiana tabacum*) microspores isolated at the phase of mitosis from 100 anthers. GP92 and GP98 are indicated

Mass spectrometric analysis of GP92 and GP98 from tobacco microspores

For both GP92 and GP98, analysis based on peptide fingerprinting was not successful. Therefore their partial sequences were determined by μ-HPLC/nano-ESI–MS/MS followed by manual interpretation of CID (collision-induced dissociation) spectra. Seven sequences were obtained for GP92 and 11 for GP98. The sequences obtained were first searched separately against the *A. thaliana* database using the MS-Pattern program at Protein prospector. The results were used for further searching against nr databases at MS-Blast2.

In GP92, seven sequences displayed homology to glycosyl hydrolase family 35 (β-galactosidase, Table 1). Using all sequences and searching at MS-BLAST2, the β-galactosidase proteins At1g31740 and At5g20710 were identified as having the highest scores.

In GP98, ten partial sequences displayed homology to various glycosyl hydrolase families (Table 2). One peptide sequence (LGFFDGDPK) was identified fully and was identical to glycosyl hydrolase family 3 (β-xylosidase), namely to At5g09730 from *A. thaliana*. When all 11 sequences were used in MS-BLAST2 searching, *Arabidopsis* β-xylosidases At5g64570, At3g19620, At5g09730 were identified as having the highest scores.

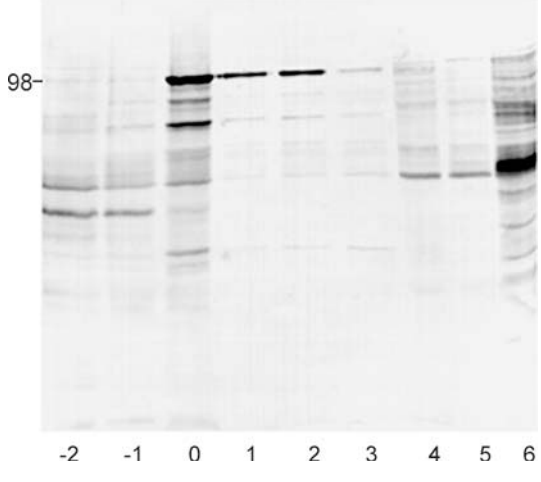

Fig. 2 ConA-binding patterns of insoluble glycoproteins during microspore and pollen development in tobacco. Proteins were extracted from early, mid-, late and mitotic microspores (*−2*, *−1*, *0*, *1*) and from different stages of pollen development (*2*, *3*, *4*, *5*, *6*). The aliquots of extracts loaded on the gel corresponded to the amounts of microspores/pollen in 50 (*−2*, *−1*, *0*), 10 (*1*, *2*, *3*) and 5 (*4*, *5*, *6*) anthers

Table 1 Summary of peptide sequences of the 92-kDa glycoprotein (GP92) from tobacco (*Nicotiana tabacum*), as determined by manual interpretation of CID spectra after μ-HPLC/nano-ESI–MS/MS. The sequences were searched against the *Arabidopsis thaliana* database using the MS-pattern program at Protein Prospector. The amino acid substitution in the matched sequence is in *boldface and underlined*. All peptides showed homology to glycosyl-hydrolase family 35 (β-galactosidase)

Sequence obtained	Matched sequence	Locus name
VLLSGSLHYPR	VLLSGS**I**HYPR	At1g31740
	VL**I**SGS**I**HYPR	At5g20710
	ILLSGS**I**HYPR	At4g26140
	ILLSGS**I**HYPR	At5g56870
	ILLSGS**I**HYPR	At1g45130
QYDFSGNLDLLR	QYDFSGNLDL**I**R	At1g31740
	EYDFSGNLD**V**VR	At5g20710
QYDFSGNLDDVR	QYDFSGNLD**L**I**R**	At1g31740
TLQDEGLYAVLR	T**I**QD**A**GLY**S**V**L**R	At5g20710
MWTENWTGWFK	MWTENWTGWFK	At1g20710
	MWTENWTGW**Y**K	At5g31740
TAEDVAFSVAR	TAED**L**AFSVAR	At1g20710
	TT**T**EDVAFA**A**VAR	At5g31740
	P**A**EDM**A**FSVAR	At3g13750
YGHLKQLHDVLHAMEK	YGHLKQLHDVLHAMEK	At1g31740
	YGHLKQLHDV**F**HAMEK	At2g04060

Activities of β-galactosidase and β-xylosidase in tobacco microspores and pollen at various stages of development

Developmental changes in β-galactosidase and β-xylosidase activity in microspores and pollen were determined as the amount of released *p*-nitrophenol from *p*-nitrophenyl-β-D-galactopyranoside or *p*-nitrophenyl-β-D-xylopyranoside. The activity of β-galactosidase increased sharply during microspore development, reached its maximum of 18.4 units at and shortly after microspore mitosis, and then fell rapidly to only about 2 units during the late stages of pollen maturation (Fig. 3). Similarly the activity of β-xylosidase showed a maximum of 6.4 units at microspore mitosis and almost zero activity in early and mid-microspores and in maturing pollen (Fig. 3).

Expression profiles of glycosyl hydrolase families 35 and 3 during microspore and pollen development in *Arabidopsis*

Pollen and sporophyte transcriptome data allowed the extraction of expression profiles for glycosyl hydrolase families 35 and 3. *Arabidopsis* glycosyl hydrolase family 35 has 18 members (http://afmb.cnrs-mrs.fr/CAZY), three of which (At2g16730, At4g35010, At5g20710) are pollen specific (Table 3). β-Galactosidase At5g20710 exhibited the highest expression in microspores and young bicellular pollen. A substantially lower expression was observed for β-galactosidases At1g45130, At1g72990, At1g77410, At2g284740, At2g32810, At3g52840, At4g38590, At5g63800 and At5g63810. High expression was confirmed for two late pollen-specific β-galactosidases (At4g35010, At2g16730) in tricellular and mature pollen. Relatively high expression in mature pollen was also observed for At5g56870. The calculated molecular mass of the early β-galactosidase gene At5g20710 is similar to tobacco GP92. The large number of potential N-glycosylation sites (10) of this β-galactosidase (Table 4) is consistent with the affinity of GP92 for lectin ConA (Hrubá and Tupý 1999).

A phylogenetic tree clearly showed that the early (At5g20710, At1g31740) and the late (At2g16730, At4g35010) β-galactosidase genes of *Arabidopsis* form distinct subgroups. β-Galactosidase Q9FSF9 expressed in mature tobacco pollen is a late pollen gene (Rogers et al. 2001). β-Galactosidase At5g56870 expressed in

Table 2 Summary of peptide sequences of the 98-kDa tobacco glycoprotein (GP98) obtained by μ-HPLC/nano-ESI–MS/MS followed by manual interpretation of CID spectra. The sequences were searched against the *A. thaliana* database using the MS-pattern program at Protein Prospector. The amino acid substitution in the matched sequence is in *boldface and underlined*

Sequence obtained	Matched sequence	Protein name	Locus name
LGFFDGDPK	LGFFDGDPK	Glycosyl hydrolase family 3 (β-xylosidase)	At5g09730
GDTAFGVAR	**E**D**L**AFGVAR	Glycosyl hydrolase family 35 (β-galactosidase)	At4g36360
PV[I/L][I/L]K	PV**I**I**K**	Glycosyl hydrolase family 9 (endo-1,4-β-glucanase)	At4g11050
GNADQA[IL]E	**S**NADQAIE	Glycosyl hydrolase family 1 (β-glucosidase)	At3g60120
[I/L]GFFDD	**L**GFFD**G**	Glycosyl hydrolase family 3 (β-xylosidase)	At5g09730
LAFGVAR	LAFGVAR	Glycosyl hydrolase family 35	At4g36360
GHETPGED	G**Q**ETPGED	Glycosyl hydrolase family 3	At1g02640
[I/L]WAGYP	IWAGYP	Glycosyl hydrolase family 3	At1g78060At5g49360
DGEPK	DGEPK	Glycosyl hydrolase family 36 (alpha-galactosidase)	At5g40390
SSAGGGG	SS**S**GGGG	Glycosyl hydrolase family 17 (endo-1,3-β-glucanase)	At5g42100
FSVQVPG	FS**S**QVPG	Glycosyl hydrolase family 3	At5g64570
AT[I/L]VFGSYD	A**A**L**V**K**G**SYD	Glycosyl hydrolase family 1	At5g26000

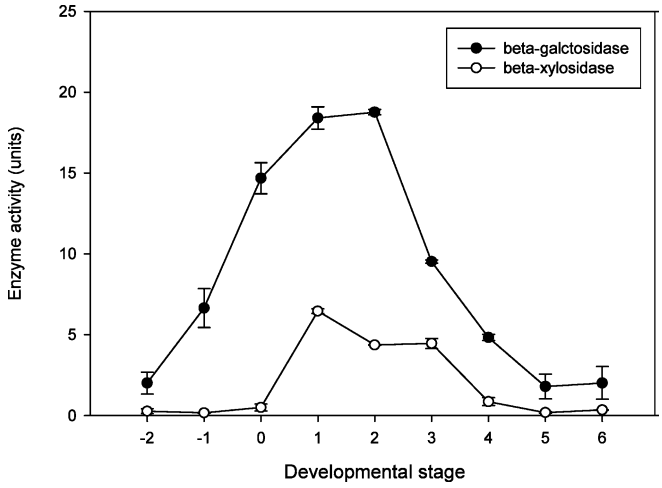

Fig. 3 *β*-Galactosidase and *β*-xylosidase activities in the extracts of early, mid-, late and mitotic microspores (*−2*,*−1*, *0*, *1*) and of early (*2*), mid- (*3*, *4*), maturing (*5*) and almost mature (*6*) pollen of tobacco. One unit of *β*-galactosidase or *β*-xylosidase activity is defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per minute. Means ± SE of three experiments expressed per microspore or pollen of 50 anthers

mature pollen and also in the sporophyte (Table 3) is more closely related to the early genes than to the late gene subgroup (Fig. 4).

Glycosyl hydrolase family 3 in *A. thaliana* has 15 members (http://afmb.cnrs-mrs.fr/CAZY). From these only one *β*-xylosidase, At3g62710, is highly expressed in pollen (Table 3). In contrast to tobacco GP98 expressed in late microspores and young pollen, the expression of pollen-specific *β*-xylosidase At3g62710 reaches its maximum in mature pollen (Table 3). The level of early *β*-xylosidase genes expression revealed by microarray analysis was very low (At5g09730, At5g10560 and At5g49360); of these the highest expression was found for At5g10560. The calculated molecular mass (87.1 kDa) of *β*-xylosidase At5g10560 (Table 5) is, however, lower than that of the early tobacco *β*-xylosidase (98 kDa).

Verification by RT–PCR of *β*-galactosidase and *β*-xylosidase expression data obtained from 23 K GeneChip experiments

The expression profiles of *β*-galactosidase and *β*-xylosidase genes during *Arabidopsis* microspore and pollen development were verified by RT–PCR analysis of RNA isolated from different stages of pollen development and from various parts of the sporophyte. RT–PCR primers were designed for early (At5g20710) and late (At2g16730, At4g35010, At5g56870) *β*-galactosidase genes and for three *β*-xylosidase genes (At3g62710, At5g10560, At5g47000). As *β*-galactosidase At1g31740 with high homology to GP92 was not present on the 23 K GeneChip we also designed RT–PCR primers for this gene.

The results confirmed our presumption that all five abundant *β*-galactosidase genes are expressed in microspores and/or pollen. Two of them (At5g20710 and At1g31740) were expressed at maximum level in the early stages of development and three (At4g35010, At2g16730 and At5g56870) in mature pollen (Fig. 5).

A strong expression of *β*-xylosidase At3g62710 in tricellular and mature pollen and very weak expression of *β*-xylosidase At5g10560 in microspores was consistent with the results of microarray analysis. A weak signal for *β*-xylosidase At5g47000 found by microarray analysis was not confirmed by RT–PCR.

Discussion

Microgametogenesis involves several distinct phases of development. Studies of the mechanism regulating this development have focused on the analysis of developmental patterns of gene expression and the isolation of mutants affecting development (McCormick 1993; Twell 2002). In this respect it was of interest to identify genes for two abundant glycoproteins, GP92 and GP98, characteristic of tobacco microspores at the phase of mitosis (Říhová et al. 1996; Hrubá and Tupý 1999).

MS analysis and database searching revealed that the cytosolic GP92 showed homology to *β*-galactosidases At1g31740 and At5g20710, and that GP98 associated with the insoluble microspore fraction displayed homology to *β*-xylosidases At5g64570, At3g19620, At5g09730 from *Arabidopsis*. The identification of GP92 as *β*-galactosidase and GP98 as *β*-xylosidase was supported by assays of the enzymes in microspore and pollen extracts. The *β*-galactosidase and *β*-xylosidase activities displayed similar developmental patterns with peak expression about the time of microspore mitosis, as observed for the occurrence of the two glycoproteins.

High activity of *β*-galactosidase in tobacco at the stage of mitosis was also revealed cytochemically but no protein or significant levels of a transcript homologous to *β*-galactosidase were reported for this developmental stage (Rogers et al. 2001). The gene Q9FSF9 with significant homology to *β*-galactosidase isolated from a cDNA library of mature pollen was expressed in mid to late pollen and the putative protein Q9FSF9 had a predictive molecular mass of 81.3 kDa (Rogers et al. 2001). Expression of late genes for *β*-galactosidase in the male gametophyte was reported also in species with tricellular pollen on the basis of microarray analysis of the transcriptome from mature pollen in *Arabidopsis* (Becker et al. 2003; Honys and Twell 2003) and of sequence analysis of proteins extracted from rice anthers (Tursun et al. 2003). In *Brassica campestris*, cytochemical assays showed maximal activity of the enzyme in pollen at the stage of generative cell mitosis (Singh et al. 1985). Transcriptomic data analysis showed abundant expression of three late *β*-galactosidase genes (At2g16730, At4g35010 and At5g56870) and of one early *β*-galactosidase gene (At5g20710). RT–PCR anal-

Table 3 Expression profiles of β-galactosidase and β-xylosidase gene family in *Arabidopsis*, adapted from publicly available microarray database (http://Arabidopsis.info/prototype, for details see <crossref seeheading ="Sec4">Materials and methods</crossref>). Genes At1g31740 and At5g04885 were not present on the 23 K GeneChip. *UNM* Uninucleate microspore,*BCP* bicellular pollen, *TCP* tricellular pollen, *MPG* mature pollen grain,*INF* inflorescence,*COT* seedlings,*LEF* leaves,*PET* petiole,*STM* stem base,*ROT* root,*RHR* root hair zone,*SUS* suspension culture.

	UNM	BCP	TCP	MPG	INF	COT	LEF	PET	STM	ROT	RHR	SUS
β-Galactosidase												
At1g45130	0	44.7	0	0	715.2	339.9	102.7	441.7	81.8	654.9	1070.4	0
At1g31740	–	–	–	–	–	–	–	–	–	–	–	–
At1g72990	282.9	317.1	225.8	209.9	275.1	286.9	272.6	288.1	465.6	121.8	164.2	166.4
At1g77410	230.9	201.6	317.1	309.7	169.3	99.9	111.9	109.7	109.1	77.3	78.1	95.6
At2g04060	0	0	0	0	0	32.7	0	0	0	15.6	0	0
At2g16730	0	212.3	2,739.7	4,148.1	93.4	0	0	0	0	0	0	0
At2g28470	385.7	411.2	0	0	1,547.1	810.3	606.5	1,201.3	564.7	478.4	569	98.9
At2g32810	484.7	514.9	0	0	936.3	410.3	475.3	820.5	1,088.6	756.1	861.6	741.8
At3g13750	0	43.7	0	0	1,065	1,029.9	1,203.4	1,576.3	1,846.2	732.4	298.9	397.1
At3g52840	68.9	0	0	0	214.8	548.7	479.4	1,318.4	542.1	140.4	0	0
At4g26140	0	0	0	0	104.5	120.8	0	182.3	0	109.2	198.8	0
At4g35010	234.8	1,176	4,706.9	5,961.1	339.8	0	0	0	0	0	0	0
At4g36360	0	0	0	0	1,274.2	636.9	479.9	1,145.3	692.2	767.6	622.9	408.4
At4g38590	113.6	103.6	143	162.3	104.2	72.8	83.7	117.4	92.4	56.1	98.7	0
At5g20710	2,679.5	3,365.6	1,326.7	824.6	517.1	0	0	0	0	0	0	0
At5g56870	0	0	908.9	3,613.4	154.1	1,483.9	936.1	201.9	3,346.9	663.7	2,374.9	0
At5g63800	110.3	0	40.1	63.8	178.4	312.8	1,216.1	1,036.4	622.8	366.3	490.1	113
At5g63810	119.4	134.1	0	101.7	516.4	309.8	153	505.7	79.9	125.9	133.3	127.8
β-Xylosidase												
At1g02640	0	0	0	0	833.9	1,482.9	424.1	321.5	1,579.7	910.5	0	0
At1g78060	0	0	0	0	428.1	360.2	346.5	583.9	188.2	703.8	426.7	126.3
At3g19620	0	0	0	0	242.7	0	0	0	0	0	0	0
At3g47000	76.7	0	99.5	169.5	373.1	371.3	379.3	447.9	495.5	381.1	372.6	480.9
At3g47010	0	0	0	0	0	77.9	97.7	281	0	230.3	0	81.9
At3g47040	0	0	0	0	0	11.3	0	0	0	172	112.2	0
At3g47050	0	0	185.2	0	0	0	0	0	0	53.4	0	554.7
At3g62710	162.4	427.6	4,830.1	8,344.5	0	0	14.1	0	69.7	0	0	0
At5g04885	–	–	–	–	–	–	–	–	–	–	–	–
At5g09730	100.8	0	0	0	211.3	0	0	0	0	0	0	0
At5g10560	288.4	260.5	66.5	0	422.4	531.5	395.9	625.3	262.9	306	297.7	286
At5g20940	0	0	0	0	0	0	0	0	0	0	0	0
At5g20950	0	0	0	0	1,138.7	875.1	777.3	1,276.2	440.1	972.1	747.7	707.3
At5g49360	272.7	0	0	0	642.3	3,138.1	1,586.2	1,317.4	2,524.6	596.1	415.7	351.1
At5g64570	0	0	0	0	1,048.6	1,141.2	886.4	706.7	665.1	469.9	392.9	76

yses were consistent with the results of microarray analysis (Honys and Twell 2003). Moreover, a second β-galactosidase gene At1g31740 with high homology to tobacco GP92 was shown to be an early pollen gene. Thus, among pollen-specific β-galactosidase genes in *Arabidopsis*, there are two early (At1g31740, At5g20710) and two late genes (At2g16730 and At4g35010). The early expression, high homology, similar size and number of potential glycosylation sites of At1g31740 indicate close similarity to tobacco GP92. Moreover, the β-

Table 4 Characterization of β-galactosidase proteins expressed in microspores and/or pollen of *Arabidopsis*

β-Galactosidase	Computed molecular mass (kDa)	Number of potential N-glycosylation sites
At1g31740	87.4	9
At5g20710	92.5	10
At2g16730	94	9
At4g35010	95.5	8
At5g56870	80.6	1
At2g28470	93.1	4
At2g32810	99.1	5
At1g72990	78.6	6
At3g52840	81.9	1
At4g38590	112.1	5
At5g63810	83.1	7

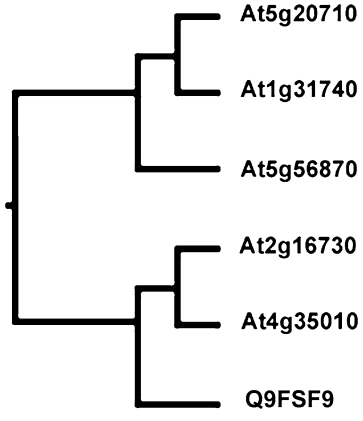

Fig. 4 Phylogenetic tree based on shared amino acid identity of tobacco pollen β-galactosidase Q9FS9 and of *Arabidopsis thaliana* β-galactosidase genes abundantly expressed early (At5g20710, At1g31740) or late (At2g16730, At4g35010 and At5g56870) during microspore and pollen development

Table 5 Characterization of β-xylosidase proteins with highest homology to peptide sequences of the 98-kDa tobacco glycoprotein (Q9FLG1, At3g19620, At5g09730) and of β-xylosidases expressed in the male gametophyte of *Arabidopsis* (At5g10560, At3g62710, At3g47000)

β-Xylosidase	Computed molecular mass (kDa)	Number of potential N-glycosylation sites
At5g64570	84.2	5
At3g19620	85.5	10
At5g09730	83.2	7
At5g10560	87.1	5
At3g62710	70.8	5
At3g47000	66.2	2

galactosidase gene Q9FSF9 reported by Rogers et al. (2001) has the highest homology to late β-galactosidase genes At2g16730 and At4g35010. A phylogenetic tree showed that the early and the late β-galactosidase genes form distinct subgroups. β-Galactosidases At1g31740 and At5g20710 of *Arabidopsis* with homology to GP92 of tobacco belong to the early group and β-galactosidases At2g16730 and At4g35010 of *Arabidopsis* with homology to tobacco Q9FSF9 form the late group. Expression of several different genes for β-galactosidases was also reported during tomato fruit ripening (Smith and Gross 2000). Similar to tomato β-galactosidases, *Arabidopsis* β-galactosidases differ in their molecular mass, the number of potential N-glycosylation sites and in their temporal expression pattern.

Our results for β-xylosidase present the first report of the expression of this enzyme during microgametogenesis. In plants, β-xylosidase activity has been reported in the culture medium of sycamore cells (Tezuka et al. 1993). A thermostable β-xylosidase has been isolated from sugarcane (Chinen et al. 1982) and two isoenzymes of β-xylosidase have been purified from immature seeds of cucumber (Mujer and Miller 1991). β-Xylosidase activity and expression of the β-xylosidase gene were also reported during strawberry fruit ripening (Martínez et al. 2004). Recently, two β-xylosidase cDNAs, designated LEXYL1 and LEXYL2, were identified that were differentially expressed during fruit development of tomato (Itai et al. 2003). The most well-characterized β-xylosidase gene in plants is AtBXL1 (At5g49360) of *A. thaliana* that encodes a putative protein of 83.5 kDa with two potential N-glycosylation sites (Goujon et al. 2003). Cell wall β-xylosidase activity was high in stems, flowers and siliques and low in seedlings and leaves. Microarray data indicate that At5g49360 coding for protein AtBXL1 is expressed at high levels in green sporophytic tissues, less in roots and suspension-culture cells and at low level in microspores. This gene is proposed to be involved in hemicellulose metabolism in secondary cell walls (Goujon et al. 2003).

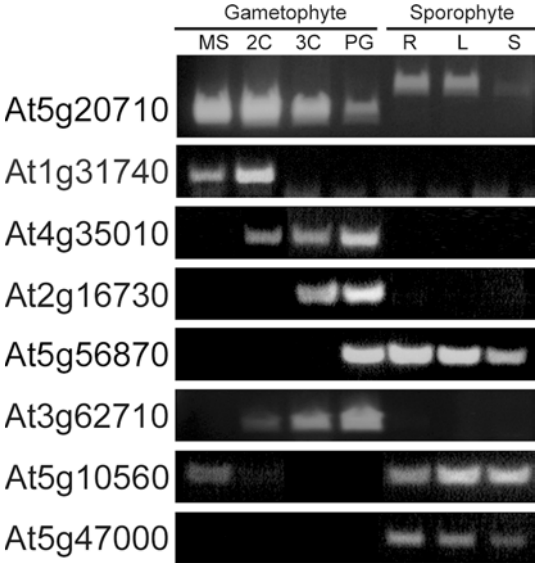

Fig. 5 Verification of expression profiles of five pollen β-galactosidase and three β-xylosidase genes in different phases of male gametophyte development (*MS* microspore, *2C* bicellular pollen, *3C* tricellular pollen, *PG* mature pollen grain) and in various parts of sporophyte (*R* root, *L* leaves, *S* stem) of *A. thaliana* by RT–PCR. A higher band in sporophytic samples of Atg20710 represents genomic DNA

The verification of the results of microarray analysis for β-xylosidase in *Arabidopsis* by RT–PCR showed one abundant pollen-specific β-xylosidase gene At3g62710 and the low expression of the early β-xylosidase gene At5g10560. The signal for the At3g47000 β-xylosidase gene revealed by microarray analysis was very weak and RT–PCR did not confirm its expression in pollen. However, the peptide sequences of GP98 showed the highest homology to β-xylosidase genes At5g64570, At3g19620 and At5g09730, the expressions of which were not detected or were very low in *Arabidopsis* pollen. This could be due to gene diversity between *Nicotiana* and *Arabidopsis* or to our inability to determine more complete sequences for GP98 by μ-HPLC/nano-ESI–MS/MS limiting the database searching.

Pollen development requires numerous modifications of the cell wall, a structure composed of pectins, hemicelluloses, celluloses and proteins. These modifications may be mediated by enzymes of pectin degradation and also by other enzymes of the glycosyl hydrolase family. Pectin-degrading enzymes include pectin esterase, polygalacturonase and pectate lyase, which are well described in pollen of various plants (reviewed in Sari-Gorla and Pé 1999; Twell 2002). β-Galactosidase and β-xylosidase belong to enzymes of the glycosyl hydrolase family. β-Galactosidase catalyzes the hydrolysis of terminal galactosyl residues from carbohydrates, glycoproteins and galactolipids. Plant β-galactosidase has an important role during fruit softening (Smith and Gross 2000). The β-xylosidase-catalyzed removal of successive D-xylose residues from the non-reducing termini of xylans, which belong to the hemicellulosic component of the primary cell wall, might also be important for cell wall elasticity. β-Xylosidase was also found to be able to release β-xylose from N-linked glycoproteins (Tezuka et al. 1993).

We suggest that early β-galactosidase and β-xylosidase genes may participate in cell wall loosening

associated with young pollen expansion after microspore mitosis, and that the function of β-galactosidase in mature pollen may be associated with pollen germination and pollen tube penetration through the style.

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