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ORIGINAL ARTICLE

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Isolation and characterization of male-germ-cell transcripts in Nicotiana tabacum

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Abstract Until recently, little knowledge existed about the molecular mechanisms regulating male gamete development. This was mainly due to the low transcriptional activity and the cellular inaccessibility of the generative and sperm cells that are enclosed by the vegetative cell in pollen. In order to study sperm cell development and possible preferential fusion during double fertilization, we have constructed a cDNA library of mRNA isolated from pure tobacco sperm cells. An initial screen of 396 clones from this library has yielded 2 cDNAs representing sperm-cell-expressed transcripts, designated NtS1 and NtS2. A preliminary characterization of these two clones showed that they accumulate in both the generative and sperm cells (i.e. the male gamete) indicating that gene expression programs between these two cell types overlap. In addition, we found that NtS1 codes for a polygalacturonase suggesting a role for this enzyme in wall degradation during differentiation of the male germ cells in tobacco. Together, these results show that with the construction of this sperm-cell cDNA library we now have a powerful tool to investigate male gamete development and function.

Keywords *Nicotiana tabacum* · Generative cell · Sperm cell · Male gamete · cDNA library

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Introduction

Development and differentiation of plant male gametes starts with an asymmetric division of the microspore, resulting in a relatively large vegetative cell and a much smaller generative cell that after one further division will give rise to two sperm cells. In tricellular pollen like that of rape (*Brassica*) seed and grasses such as maize (*Zea*) and wheat (*Triticum*), the division of the generative cell occurs in the pollen grain before maturation. In bicellular pollen like that of lily (*Lilium*) and tobacco (*Nicotiana*), the division of the generative cell occurs after dispersal of the pollen and its germination on the surface of the stigma (Russell and Dumas 1992). The switch from vegetative to generative development relates to the asymmetry of the microspore division, but the actual triggers and regulation are still unknown (Twell et al. 1998). The development of the large vegetative cell is accompanied by the expression of a number of specific genes such as *LAT52* and *LAT59* (McCormick 1991), *Zm13* (Hamilton et al. 1992), and *NTP303* (Weterings et al. 1995) that may relate to pollen maturation, germination and pollen tube growth. In contrast, the cytoplasmic volume of the generative cell is extremely small and its nucleus becomes heterochromatic, indicating little or no gene activity (Mascarenhas 1993). With the exception of *LGC1* from lily (Xu et al. 1999), no specifically expressed genes have been isolated.

Although the symmetric division of the generative cell produces two sperm cells that may differ in size and morphology, the sperm cells remain connected as a single male germ unit (Dumas et al. 1984; Russell et al. 1990). The cytoplasmic volume of the sperm cells always stays very small and the nuclei remain heterochromatic (Mogensen 1992; Twell et al. 1998; Yu et al. 1992). At fertilization, one of the two sperm cells fuses with the egg cell that then will develop into the embryo, while the other sperm cell fuses with the central cell that will form the endosperm. Until now, only the morphologically different sperm cells of *Plumbago zeylanica* have been shown to fuse preferentially with either the

egg or the central cell; sperm cells from all other species examined to date have not shown this preferential fusion (Faure 1999; Hu 1998; Russell 1992). Therefore, it is still unknown whether a general mechanism that targets sperm cells specifically to the egg and central cell exists, and how this mechanism might work.

Due to their inaccessibility and low transcriptional activity, little is known about the molecular biology of the male gamete (i.e., generative and sperm cells; Knox et al. 1993; Tanaka 1997). For example, it is not known whether the gene expression program is different between sperm cells, and between the sperm cells and the generative cell. So far, screening of cDNA libraries from pollen and pollen tubes has not provided any markers for male gamete development. However, recently developed procedures for mass isolation of generative cells and sperm cells enabled the isolation and characterization of sperm-cell-expressed genes, such as the male-gamete-specific gene *LGC1* of lily (Xu et al. 1999), as well as the identification of sperm plasma membrane proteins of *Brassica campestris*, *Lilium longiflorum*, and *Zea mays* (Blomstedt et al. 1992; Southworth and Kwiatkowski 1996; Xu and Tsao 1997).

In this paper we describe the production of a cDNA library from mass isolated sperm cells of *Nicotiana tabacum*. In order to study sperm-cell development and possible preferential fusion, we isolated two cDNA clones – NtS1 and NtS2 – representing sperm-cell transcripts and studied their developmental accumulation patterns.

Materials and methods

Plant material

Plants of *N. tabacum* L. cv. 'Petit Havana' were grown in soil under standard greenhouse conditions (15 h light/9 h dark, 18–20°C, 65% relative humidity) at the Nijmegen University Botanical Garden. Flower tissues were collected at different stages according to the morphological markers described by Koltunow et al. (1990). Mature pollen was collected from flowers at stage 12 and petals, ovaries, anthers, and pistils without ovaries were collected at stage 10 unless otherwise indicated. For pollination experiments, flowers were emasculated at stage 11 and pollinated with fresh pollen 1 day later. Pollen tubes were cultured in vitro according to Read et al. (1993), washed in 15% sucrose prior to collection, frozen in liquid nitrogen, and stored at –80°C.

Mass isolation and purification of sperm cells

Pollen tubes were cultured in vitro for 22 h, collected on 30 µm nylon filters (Merren and Laforte), and washed in 15% sucrose to remove the growth medium. To release the sperm cells, the pollen tubes were incubated in a cell-wall-degrading enzyme mixture [0.6% Cellulase (Onozuka-R10; Yakult/Honsha) and 0.3% Macerozyme (Yakult/Honsha) in 2 mM MES buffer (pH 6.7), 8% sucrose, and 0.1% BSA] for 20 min at room temperature. Burst pollen tubes were removed by filtration through four layers of 11 µm nylon mesh (Merren and Laforte) and the final sucrose concentration of the filtrate was adjusted to 17% by addition of 30% sucrose in 2 mM MES buffer (pH 6.7). This suspension was layered onto a discontinuous Percoll gradient consisting of two layers of 7.5 ml of 15% and 22% Percoll in 15% sucrose, 2 mM MES (pH 6.7), and centrifuged for 30 min at 2,700 *g* at 4°C (Xu and Tsao 1997). Sperm cells were collected from the sperm-cell-rich layer at the bottom of the 15% Percoll zone and washed twice in 15% sucrose, 2 mM MES (pH 6.7). To check yield and purity, samples were analyzed by microscopy under Nomarski differential interference contrast with a Zeiss Axiomat III microscope and by fluorescence microscopy after DNA staining with DAPI (AppliChem) in a Leitz Ortholux-orthoplan with appropriate filters and illumination. Samples were frozen in liquid nitrogen and stored at –80°C for RNA isolation. Sperm cells that were isolated for RNA to be used in RT-PCR were treated with 50 µg/ml RNase (Promega) at 37°C for 30 min prior to freezing.

Nucleic acid isolation

Quantities of 0.1–1 g of frozen tissue were homogenized in 2 ml RNA extraction buffer [0.1 M Tris (pH 8.0), 0.1 M NaCl, 1% SDS, 50 mM EDTA, 50 mM β-mercaptoethanol, and 1% tri-iso-propyl-naphtalene-sulphonic acid sodium salt (Eldik et al. 1995)] and protein was removed by phenol extraction. Total nucleic acids were precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. The resulting pellet was dissolved and RNA was precipitated with one-third volume of 8 M LiCl. RNA for RT-PCR was treated with 1 U DNase for 30 min at 37°C, extracted with phenol and precipitated with ethanol. Sperm-cell poly(A^+) mRNA was purified using the Qiagen Direct mRNA Isolation Kit according to the manufacturer's protocol. Plasmid DNA was isolated with the alkaline lysis method according to Sambrook et al. (1989).

cDNA library construction and screening

The tobacco sperm cell cDNA library was constructed following the procedure provided with the ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). Sperm-cell poly(A^+) mRNA (5 µg) was used for synthesis of the first strand cDNA. Size selected cDNA molecules (1–3 kb) were ligated into Uni-ZAP XR vectors. The primary library was amplified once prior to screening. Screening for sperm-cell specific cDNAs occurred in two rounds. In the first round the cold plaque approach (Hodge et al. 1991) was adopted to identify low abundance genes. Two duplicate plaque filters were hybridized to two different ^{32}P-labeled cDNA probes of mRNA from mature pollen and of pooled mRNA from young flowers (stage 6) and seedlings. The cDNA probes were prepared with the reverse transcriptase Superscript II kit (Gibco/BRL) using 1 µg of poly(A^+) mRNA. The hybridization temperature was 65°C and the filters were washed with 0.1× SSC, 1% SDS at 65°C. "Cold" plaques that did not hybridize to either of the two probes were selected as possible sperm cell-specific transcripts. In the second round, candidates were screened by reverse northern hybridization. pBluescript phagemids were prepared from the selected cold plaques following the manufacturer's in vivo excision protocol. The plasmids were digested with *Eco*RI and *Xho*I, run on an agarose gel and analyzed by Southern hybridization to three different ^{32}P-labeled cDNA probes: (1) sperm-cell poly(A^+) mRNA, (2) pollen poly(A^+) mRNA, and (3) pooled poly(A^+) mRNA from young flowers (stage 6) and seedlings.

RNA gel blot hybridization

Aliquots of 0.5 µg poly(A^+) mRNA or 20 µg total RNA were denatured, fractionated on denaturing 1.2% agarose gels containing formaldehyde, transferred to Hybond-N^+ nylon membranes (Amersham, UK), and hybridized to ^{32}P-labeled cDNA inserts. Hybridization was carried out overnight at 65°C in 5× SSC, 5× Denhardt's solution, 0.5% SDS and 100 µg/ml denatured sonicated herring sperm DNA. After hybridization, the filters were washed with 0.2× SSC, 0.1% SDS at 65°C and exposed – with intensifying screens – to Kodak X-OMAT film at –80°C. To verify

equal loading of RNA, the filters were stripped and probed with a tobacco 25S ribosomal probe.

RT-PCR

For reverse transcription (RT), 5 µg of DNase-treated RNA was used in a 20 µl reaction according to the manufacturer's protocol (MBI Fermentas) and for each PCR amplification, 2 µl of this RT reaction was used. The PCR reaction was carried out in 50 µl with 0.5 U *Taq* DNA polymerase (Eurogentec), 1.2 mM $MgCl_2$ and 50 pmol of each of the two sequence specific primers with the following cycle: 3 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at the optimal annealing temperatures (54.5°C for the NtS1 and 50°C for NtS2 primers), and 2 min at 72°C, followed by 7 min at 72°C. The NtS1-specific primer set was (XH3) 5′-ACCGGCCAAAGTT-GTTATTCCTC-3′ and (XH4) 5′-GCATGCTGGCGGGTTATTTT-3′, the NtS2-specific primer set was (HP7) 5′-AATCTGTTC-TCTAGCGGGTTCT-3′ and (HP8) 5′-TTTTGGGAGGAGGAC-AGGTAT-3′, and the NTP303 specific primer set was (F1) 5′-TTGCTACTTTGCCTCTCCGT-3′′ and (R1) 5′-CAGGCCC-TTGACGATACCG-3′.

Sequence analysis

Sequencing was performed by the di-deoxy chain termination method (Sanger et al. 1977) using the ABI-PRISM BigDye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The sequence data were analyzed with NCBI GenBank BLAST programs (http://www.ncbi.nlm.nih.gov; Altschul et al. 1997) and the Swiss Institute of Bioinformatics Prosite database (http://www.expasy.ch/). The GenBank accession numbers for NtS1 and NtS2 are AF248538 and AF248539, respectively.

Results

Mass isolation and purification of sperm cells

At maturity, tobacco pollen is bicellular and therefore sperm cells must be isolated from pollen tubes. In order to obtain sufficient amounts of sperm cells to isolate poly(A^+) mRNA for cDNA library construction, we used in vitro grown pollen tubes (Read et al. 1993). In our hands, after 22 h incubation at 25–26°C more than 90% of the generative cells had divided into sperm cells. The pollen tubes were burst by low osmotic shock in combination with enzymatic digestion of the wall, and sperm cells were purified by centrifugation on a discontinuous Percoll gradient (see Materials and Methods; Xu and Tsao 1997).

Based on the results of Nomarski and fluorescence microscopy, 4×10^5 sperm cells were obtained from pollen tubes cultured from 100 mg of fresh pollen. As shown in Fig. 1A, only a few impurities were present in the sperm cell preparations. The diameter of the isolated cells ranged between 5 and 9 µm and their shape was spherical as opposed to spindle-like in the pollen tubes. One percent of the cells were >8.5 µm diameter and represented either undivided generative cells or fused sperm cells (Tian and Russell 1998; Zhang et al. 1997). In order to distinguish between these two possibilities, we stained the isolated cells with DAPI. Figure 1B shows that all cells appeared to contain one nucleus, indicating that cells >8.5 µm represented undivided generative cells.

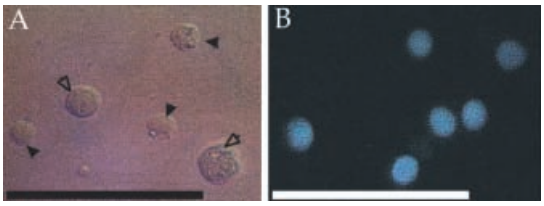

Fig. 1A, B Isolation of sperm cells. **A** Differential interference microscopy of isolated tobacco sperm cells. The small 5 µm diameter cells (*arrowheads*) represent sperm cells and the larger 9 µm diameter cells (*open arrowheads*) represent either generative cells or fused sperm cells. **B** Fluorescence microscopy of DAPI-stained isolated tobacco sperm cells. Sperm-cell nuclei appear bright blue. *Bar* 50 µm

Construction and screening of the cDNA library

We used 5 µg sperm poly(A^+) mRNA to construct a cDNA library. The final yield of clones from the primary and amplified library was 4.0×10^5 and 1.8×10^{12}, respectively, and the insert sizes ranged from 0.6 to 2 kb. Putative male-germ-cell-specific cDNA clones were identified using the "cold plaque" approach (Hodge et al. 1991). Assuming that male-gamete transcripts have a low abundance (Mascarenhas 1993) and therefore are highly diluted in the mature pollen mRNA pool, we selected plaques that generated no hybridization signal with a mature pollen cDNA probe and in addition did not hybridize to a flower and seedling mixed cDNA probe. From a total of 396 plaques in the first screening round, we thus identified 78 "cold" plaques. The cDNA clones from these plaques were subjected to a second screening by reverse northern hybridization using cDNA probes from poly(A^+) mRNA of sperm cell, pollen, and pooled young flowers and seedlings (see Materials and Methods). Based on their hybridization patterns, the 78 clones could be divided in three subclasses: (1) 53 clones that showed hybridization signal with all three cDNA probes and were therefore excluded from further analysis, (2) 8 clones that showed hybridization signal with sperm cell and pollen cDNA probes but not with the pooled flower and seedling cDNA probe, and (3) 17 clones that did not show hybridization signal with any of the three cDNA probes. The eight cDNAs from group two were further analyzed by hybridization to northern blots and we found one clone, designated NtS1, that hybridized to sperm cell, pollen, and anther RNA but not to RNA from other flower parts or vegetative plant organs (data not shown). The 17 clones from group three were further analyzed by RT-PCR and we found that only the primer set derived from clone NtS2 produced PCR products of the expected size with reverse transcribed RNA from sperm cell, pollen, and anther, although not with reverse transcribed RNA from other flower parts or vegetative plant organs (data not shown). The NtS1 and NtS2 cDNA clones were studied further by nucleotide sequence analysis, northern blot hybridization, and RT-PCR.

The nucleotide sequence of the NtS1 cDNA clone is 1,315 bp in length and its longest open reading frame

A

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1   I M A I P K E L L V L L L G I A I L S K V A E C
    cATAATGGCTATTCCAAAAGAATTATTGGTTTTACTTTTGGGTATAGCCATTTTGTCAAAAGTTGCTGAATGT  73
25  A A F G S R R G L V E T V F D V T R F G A R P N
    GCAGCTTTTGGTTCACGTCGTGGCCTTGTCGAGACAGTTTTTGATGTTACCAGGTTTGGAGCTAGACCAAAT  145
49  T P A D S T R G L M M A W R S A C Q S P G P A K
    ACCCCTGCAGATAGCACTAGGGGACTTATGATGGCATGGAGATCAGCTTGCCAATCACCAGGACCGGCCAAA  217
73  V V I P P G T Y T T G E T I F Q G P C T S P R P
    GTTGTTATTCCTCCAGGAACATACACTACAGGAGAAACTATATTTCAAGGGCCATGCACTAGTCCTAGGCCA  289
97  I T I E I Q G T L L S N T D M S L Y T R A A W I
    ATTACTATTGAAATTCAAGGAACTTTGTTGTCAAATACTGACATGAGTTTGTACACAAGAGCAGCTTGGATT  361
121 S I E H V D G I V I T G G G T L N G Q G K S S W
    TCTATAGAACATGTAGATGGCATCGTTATTACGGGTGGAGGAACTCTAAATGGACAAGGAAAATCGAGTTGG  433
145 Q F A D K S G S T P P L P S S L V F Q T V R S S
    CAATTTGCTGATAAATCTGGAAGTACCCCTCCTCTCCCCTCTTCTCTTGTATTTCAGACAGTGCGAAGTTCT  505
169 S I K N L N F V D A K G V H L K I T D S S D I S
    TCGATCAAGAACCTCAATTTTGTGGATGCCAAAGGTGTCCACTTGAAAATCACCGATAGCAGTGACATCTCA  577
193 V S N I K I T A P G D S P N T D G L H I S D T I
    GTTTCAAATATCAAAATTACAGCTCCAGGAGATAGTCCAAACACTGATGGTCTTCATATTAGTGATACAATT  649
217 N V N V T D S T I G T G D D C I S I G D G N S N
    AATGTCAATGTCACTGATAGCACCATTGGAACAGGTGACGATTGCATCAGTATTGGAGATGGCAATTCTAAT  721
241 V Y I S N I N C G P G H G I S I G S L G K R L D
    GTTTATATCTCTAACATAAATTGTGGCCCGGGACATGGAATAAGCATCGGCAGCCTTGGGAAAAGGTTAGAT  793
265 E T D V K G V T V R N C T F R G T T N G A R I K
    GAAACGGACGTTAAAGGAGTTACAGTAAGAAACTGTACATTCCGTGGTACAACAAATGGTGCAAGAATCAAA  865
289 T Y M G S P S L Q V S G V V Y E D I I L D N V K
    ACTTATATGGGCTCACCTTCACTACAAGTTTCTGGTGTTGTTTATGAAGATATAATCCTGGATAATGTTAAA  937
313 N P I V I D Q H Y H S K N R N E P S R V K L V D
    AATCCCATAGTTATTGATCAACACTATCATTCCAAGAACAGGAATGAGCCATCTAGAGTGAAACTGGTGGAT 1009
337 I H F R N I R G T T T S K I P V L L N C S E A L
    ATTCACTTCAGGAACATAAGGGGAACAACAACTTCAAAAATTCCAGTGTTACTCAATTGCAGTGAAGCACTT 1081
361 P C E G V E L V D I D L A P V G S I G P L L P A
    CCCTGTGAAGGAGTTGAATTGGTTGACATTGATCTAGCTCCAGTTGGTTCAATTGGACCTCTTTTGCCTGCA 1153
385 T C Q N A K T I L N G K N N P P A C ●402
    ACTTGTCAAAATGCCAAAACAATATTGAATGGAAAAAATAACCCGCCAGCATGCTGAttaattattagtacg 1225
    tcttttctacctcaaattatgtctatgaaaaatgtatatatatcctgatttaatcacataatttgcttaaag 1297
    gt(a)n 1315
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B

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1   Y I G I C S L A G S L T V M G V K A I G I A M
    tcTACATTGGAATCTGTTCTCTAGCGGGTTCTCTCACGGTTATGGGTGTCAAAGCAATTGGAATTGCTATG   71
24  K L T F G G Q N Q F K Y F E T W F F I I F V L I
    AAGCTCACATTTGGAGGACAAAATCAATTCAAGTATTTTGAGACATGGTTTTTCATAATATTTGTCCTCATC  143
48  F C L L Q L N Y L N K A L D T F N T A V V S P I
    TTTTGCCTTTTGCAGCTGAACTATTTGAACAAGGCACTTGACACATTTAATACTGCCGTGGTTTCTCCTATA  215
72  Y Y V M F T T L T I V A S M I M F K D Y V H Q N
    TACTACGTCATGTTTACAACACTGACCATTGTTGCAAGTATGATAATGTTTAAGGACTATGTTCATCAAAAT  287
96  A T Q I I T E L C G F V T I L C G T F L L H K T
    GCAACACAGATAATTACAGAGTTATGTGGTTTTGTAACTATCCTATGTGGTACTTTTCTTCTTCACAAAACC  359
120 K D M G S N P S K P I P V L L P K T D T D C K P
    AAAGACATGGGAAGCAATCCATCCAAACCAATACCTGTCCTCCTCCCAAAAACAGACACGGATTGTAAGCCA  431
144 T S E T T K I P A E V ●154
    ACAAGCGAAACAACAAAAATCCCAGCTGAGGTTTGAaggcaaaacgactaggagattgttctttaattgcat  503
    catcaagagagcatagggggaaagggagaggtgcataaagaagaagatagaagcatttgttattggataaaac  575
    ttaaggagacaagcttagcattggaaagaggtgatgttttttgacaggttgaagccaatgctatactaggaa  647
    ggtatttcgttatgattcttttttgttattttggctttagaaagattttacagtgggatactgatatcgaaa  719
    ttgacatgcttcttggaatt(a)n  757
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(ORF) is 1,209 bp translating into a 402 residue amino acid sequence (Fig. 2A). Sequence analysis and database homology searches indicated that the predicted NtS1 amino acid sequence contains a polygalacturonase active site and shares 40% identity at the amino acid level to a polygalacturonase (Tebbutt et al. 1994) that catalyzes the random hydrolysis of 1,4-β-D-galactosiduronic linkages in pectate and other galacturonans (Collmer and Keen 1986). The NtS2 nucleotide sequence is 757 bp in length and its longest ORF is 465 bp corresponding to an amino acid sequence of 154 residues (Fig. 2B). Sequence analysis and database homology searches indicated that the predicted NtS2 amino acid sequence (Fig. 2B) shares 65% similarity to the hypothetical *Arabidopsis* protein At3g23860 of unknown function (Kaneko et al. 2000).

NtS1 and NtS2 RNAs accumulate in pollen and in sperm cells

We tested the tissue-specific transcript accumulation pattern of NtS1 by RNA gel blot hybridization. Figure 3A shows that the NtS1 probe hybridized to a 1.5 kb transcript present in sperm cells and to a 1.6 kb transcript of anther and mature pollen. This probably indicated that a transcript from a different polygalacturonase gene family member was present in our sperm cell RNA preparation compared to anther and mature pollen (Tebbutt et al. 1994). No NtS1 hybridization signal was detected in lanes containing leaf, stem, petal, stigma, and ovary RNA. The lanes containing sperm cell and anther RNAs showed approximately equal hybridization signal levels, and the signal level in the pollen RNA lane was 4–5 times higher. Hybridization with a 25S-rRNA-specific probe indicated equal loading.

Because RNA from our sperm cell preparations still might contain residual amounts of vegetative cell RNA, we could not conclude from the data shown in Fig. 3A that NtS1 transcripts are present in the sperm cells. In order to remove any traces of vegetative cell RNA, we treated the sperm cell suspension with RNase prior to RNA isolation (see Materials and Methods). The effectiveness of this treatment was tested by RT-PCR using the pollen-specific mRNA NTP303 as a marker for the presence of vegetative cell RNA (Weterings et al. 1995). Figure 3B shows that an NTP303 PCR product was amplified from RNA from untreated sperm cell preparations. In contrast, no NTP303 PCR fragment was amplified from RNA isolated from RNase-treated sperm cells indicating that vegetative cell RNA had been removed.

Fig. 2A, B NtS1 and NtS2 cDNA nucleotide sequences and amino acid sequences of their longest open reading frames. The amino acid sequences are numbered on the left and the nucleotide sequences are numbered on the right. The predicted coding regions are in *upper case* and the non-coding regions are in *lower case*. In the amino acid sequence of NtS1, the polygalaturonase active site is in *bold* and the *N*-linked glycosylation sites are *underlined*. **A** NtS1, **B** NtS2

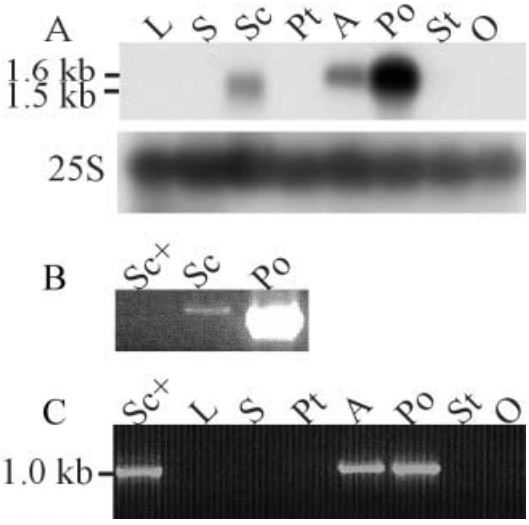

Fig. 3A–C RNA gel blot and RT-PCR analysis of NtS1 transcripts. **A** RNA gel blot analysis of NtS1 transcript accumulation in different tissues. Each lane contained 20 µg total RNA. Blots were sequentially hybridized to an NtS1 probe (*upper panel*) and a tobacco 25S rRNA probe (*lower panel*). Exposure times: NtS1 probe, 6 days at –80°C with intensifying screens; 25S rRNA probe, 20 h. **B** RT-PCR analysis of the presence of vegetative cell specific NTP303 transcripts in RNA from RNase-treated sperm cells. **C** RT-PCR analysis of NtS1 transcript accumulation in different tissues. *A* Anthers, *L* leaves, *O* ovaries, *Po* pollen, *Pt* petals, *S* stems, *Sc* sperm cells, Sc^+ RNase-treated sperm cells, *St* stigmas and styles

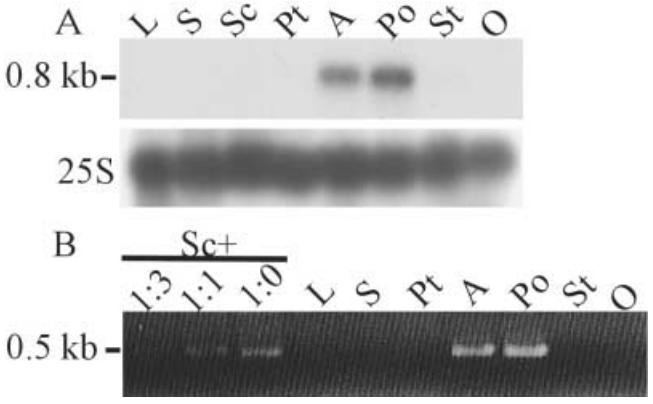

Fig. 4A, B RNA gel blot and RT-PCR analysis of NtS2 transcripts. **A** RNA gel blot analysis of NtS2 transcript accumulation in different tissues. Each lane contained 20 µg total RNA. Blots were sequentially hybridized to an NtS2 probe (*upper panel*) and a tobacco 25S rRNA probe (*lower panel*). Exposure times: NtS2 probe, 30 days at –80°C with intensifying screens; 25S rRNA probe, 20 h. **B** RT-PCR analysis of NtS1 transcript accumulation in different tissues. *1:0*, *1:1*, *1:3* Dilutions of the mRNA input, *A* anthers, *L* leaves, *O* ovaries, *Po* pollen, *Pt* petals, *S* stems, *Sc* sperm cells, Sc^+ RNase treated sperm cells, *St* stigmas and styles

Using this RNA in an RT-PCR with NtS1-specific primers generated a 1.0 kb product, which indicated that the sperm cell RNA was not degraded by the preceding RNase treatment. A similar 1.0 kb fragment was amplified from anther and mature pollen RNA. No PCR product was detected with the leaf, stem, petal, and stigma RNA templates (Fig. 3C), thus confirming the results obtained by RNA gel blot analysis (Fig. 3A). Together, these data show that NtS1 RNA accumulates in the sperm cells of tobacco pollen tubes 20 h after germination. The higher NtS1 hybridization levels with transcripts from mature pollen before germination and from flower-stage-10 anthers may have resulted from higher transcript levels in either the generative cell or both the generative cell and the vegetative cell.

The RNA gel blot hybridization data shown in Fig. 4A show that NtS2 transcripts were detected in the anther and pollen RNAs but not in RNAs from sperm cells, leaf, stem, petal, stigma or ovary. Figure 4B, however, shows that NtS2 transcripts were detected using RT-PCR in vegetative-cell-free sperm cell RNA and also, as expected, at higher levels in anther and pollen RNAs. No NtS2 transcripts were present in leaf, stem, petal, stigma or ovary RNA. Results from the serially diluted sperm-cell template indicated the semi-quantitative nature of these RT-PCR reactions. Together, these results show that NtS2 RNA accumulates to a very low level in the sperm cells of tobacco pollen tubes at 20 h after germination. In mature pollen, the higher NtS2 transcript abundance may be the result of higher abundance either in the generative cell or in both the generative and vegetative cell.

We studied the developmental RNA accumulation patterns of the *NtS1* and *NtS2* genes by RNA gel blot hybridization and RT-PCR, respectively (Figs. 5 and 6). Figure 5A shows that the NtS1 probe did not hybridize to RNA isolated from flower-stage-3 anthers and stage 6 anthers. NtS1 hybridization signal was first detected in mature pollen RNA. The signal level remained the same in imbibed pollen and pollen tubes grown in vitro for 2 and 6 h and NtS1 hybridization levels decreased in 12–20-h-germinated pollen. In the latter, the NtS1 probe detected a 1.5 kb transcript similar in size to the one detected previously in sperm cells (Fig. 3A). This indicates that in in vitro-germinated pollen a different polygalacturonase gene family member (Tebbutt et al. 1994) is present at the time the generative cell starts to divide. Figure 5B shows a similar NtS1 hybridization pattern for pollen tubes grown in vivo in the style. NtS1 transcript levels decreased steadily at 12, 20 and 30 h after pollination until at 34 h after pollination no NtS1 transcripts were detected.

Figure 6 shows the results of the RT-PCR analysis of NtS2 transcript accumulation during development. NtS2 transcripts start to accumulate in flower-stage-3 anthers and levels increase until maturity. From mature pollen stage onwards, NtS2 transcript levels remained the same in imbibed pollen and in vitro grown pollen tubes at 2, 6, 12 or 20 h after germination (Fig. 6A). Figure 6B shows that NtS2 transcripts were detected using RT-PCR in in vivo-grown pollen tubes in the style until 34 h after pollination. Together, these data show that both NtS1 and NtS2 RNAs accumulate to their highest levels at pollen maturity and that their levels slowly decrease after pollen germination and pollen tube growth in the style.

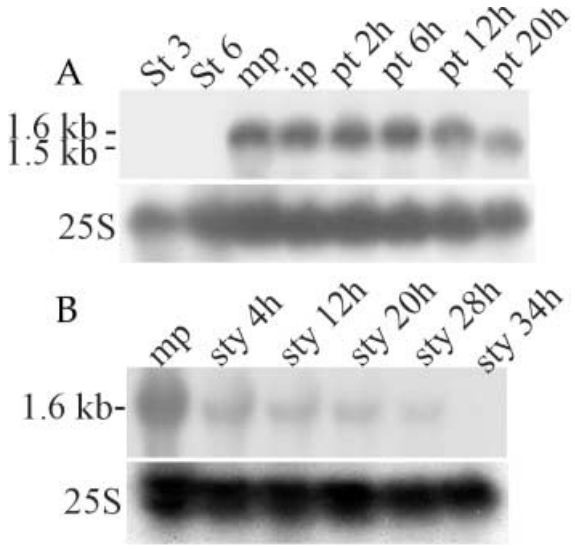

Fig. 5A, B RNA-gel blot analysis of NtS1 transcripts at different pollen developmental stages. **A** NtS1 transcript accumulation in developing and in vitro-germinated pollen. Exposure times: NtS1 probe (*upper panel*), 16 days at –80°C with intensifying screens; 25S rRNA probe (*lower panel*), 20 h. **B** NtS1 transcript accumulation in pollinated stigmas and styles. Exposure times: NtS1 probe (*upper panel*), 16 days at –80°C with intensifying screens; 25S rRNA probe (*lower panel*), 20 h. Each lane contained 20 µg total RNA. Blots were sequentially hybridized to an NtS1 probe and a tobacco 25S rRNA probe. *ip* Pollen imbibed for 30 min, *mp* mature pollen, *pt 2h*, *pt 6h*, *pt 12h*, and *pt 20h* pollen tubes grown in vitro for 2, 6,12, and 20 h, respectively; *St 3* and *St 6*, anthers from flower stages 3 and 6, respectively; *sty 4h*, *sty 12h*, *sty 20h*, *sty 28h*, and *sty 34h* stigmas and styles at 4, 12, 20, 28, and 34 h after pollination, respectively

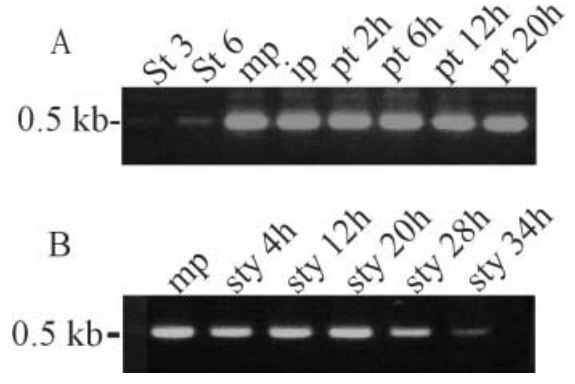

Fig. 6A, B RT-PCR analysis of NtS2 transcripts at different pollen developmental stages. **A** NtS2 transcript accumulation in developing and in vitro-germinated pollen. **B** NtS2 transcript accumulation in pollinated stigmas and styles. *ip* Pollen imbibed for 30 min, *mp* mature pollen; *pt 2h*, *pt 6h*, *pt 12h*, and *pt 20h* pollen tubes grown in vitro for 2, 6,12, and 20 h, respectively; *St 3* and *St 6*, anthers from flower stages 3 and 6, respectively; *sty 4h*, *sty 12h*, *sty 20h*, *sty 28h*, and *sty 34h* stigmas and styles at 4, 12, 20, 28, and 34 h after pollination

Discussion

Sperm cells can be mass isolated and used to construct a conventional cDNA library

To isolate and characterize genes expressed preferentially in male gametes, we used mass-isolated sperm cells from tobacco to extract and purify mRNA for the construction of a cDNA library. Sperm cells were freed by osmotic shock (Xu and Tsao 1997) in combination with enzymatic wall digestion. After concentration by centrifugation over a discontinuous Percoll gradient, this resulted in a pure sperm-cell preparation based on microscopic observations (Fig. 1). Although nanograms of RNA from a few cells may be adequate to produce cDNA libraries by PCR (Don et al. 1993; Dresselhaus et al. 1994), we used 5 µg poly(A^+) mRNA from this sperm cell prepa-ration to construct a conventional, non-PCR-based library that is strongly enriched for sperm cell cDNAs. Because the resulting library contained 4.0×10^5 pfu, and because pollen grains contain 20,000 to 24,000 different mRNAs (Mascarenhas 1990), of which a minor fraction belongs to the transcriptionally quiescent male gametes (McCormick 1993), this library probably represents all sperm cell transcripts.

Cold plaque screening yields candidate cDNA clones representing male gamete transcripts

As a primary step in the selection procedure for putative male germ cell cDNAs we used the “cold plaque” screening method (Hodge et al. 1991). We selected plaques that gave no hybridization signal with either the pollen cDNA probe or the flower and seedling mixed cDNA probe. This selection criterion was based on the rationale that generative cell cDNA probably represented a very small part of the pollen cDNA probe because the generative cell is relatively inactive (Mascarenhas 1990) and at maturity the volume is 100 times smaller than the vegetative cell (J. Derksen, unpublished data). Consequently, cDNA clones meeting the primary screening selection criteria should either represent RNAs accumulating specifically in the male gamete or RNAs accumulating at low levels in the male gamete and in the vegetative cell and/or in the flower and vegetative tissues. Results from the more sensitive secondary screening and from the characterization of NtS1 and NtS2 (Figs. 3, 4) showed that this assumption was correct. Therefore, it can be concluded that male gamete cDNAs can be isolated from the sperm cell cDNA library by using a pollen cDNA probe. In future primary screenings for male gamete cDNAs, this will allow us to circumvent tedious sperm cell isolations.

Sperm cells are transcriptionally active and share overlapping gene expression programs with generative and/or vegetative cells

The cDNA clones NtS1 and NtS2 described in this paper represent mRNAs that accumulate in the male germ cell.

We were able to demonstrate this unequivocally by using sperm cell RNA that was free of vegetative cell RNA (Fig. 3B) in the RT-PCR analyses of the NtS1 and NtS2 transcript accumulation patterns (Figs. 3C, 4B). This shows that, contrary to what was previously believed (McCormick 1993), male germ cells are transcriptionally active. In addition, the recently identified male gametic gene *LGC1* from lily (Xu et al. 1999) indicates that transcriptional activity of the male germ cell is conserved between monocots and dicots.

NtS1 and NtS2 transcripts were detected at a low level in sperm RNA and at a higher level in pollen RNA (Figs. 3A, 4B). This can be explained by: (1) higher accumulation levels of NtS1 and NtS2 transcripts in the generative cell of the mature pollen, (2) by NtS1 and NtS2 accumulation in the vegetative cell, or (3) higher accumulation in both the generative and vegetative cell. To determine which of these possibilities is correct, additional in situ hybridization studies will be necessary. However, regardless of what scenario might be true, it is clear from the data presented in this report that gene expression programs between the sperm cell and the generative cell and/or the vegetative cell overlap and that NtS1 and NtS2 transcript levels change during pollen development within these overlapping programs (Figs. 5 and 6).

NtS1 might be involved in sperm cell wall degradation

At formation, the generative cell in tobacco becomes surrounded by a cell wall that disappears gradually with maturation (Cresti et al. 1987). A similar wall was also observed in orchids (Heslop-Harrison 1968) and *Impatiens* (Cresti et al. 1987). In the tricellular pollen of wheat, the differentiating sperms are surrounded by a wall that becomes discontinuous at maturation (Hu et al. 1981; Zhu et al. 1980). In tobacco (Yu and Russell 1994; Yu et al. 1992), but also in *Plumbago zeylanica* (Russell and Cass 1981), the pair of sperm cells is interconnected by a transverse cell wall. We found that the NtS1 amino acid sequence has high similarity to a polygalacturonase that hydrolyses glycosidic linkages in pectic polymers, a primary component of plant cell walls (Collmer and Keen 1986). Therefore, we suggest that NtS1 is involved in wall degradation during differentiation of the male germ cells in tobacco.

It remains to be established whether this is the actual function of NtS1 and what function NtS2 performs in the male gamete. However, it is clear that from this sperm-cell cDNA library we can isolate male gamete mRNAs that can serve as an entry point to investigate male gamete development and function.

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