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Molecular characterization of the pollen-specific genomic clone NTPg303 and in situ localization of expression

Abstract The localization of transcripts of the pollen-specific gene NTP303 has been determined by means of in situ hybridization in combination with confocal laser scanning microscopy. NTP303 transcripts were first detectable at the mid-bi-nucleate stage of pollen development and persisted even after the pollen tube had reached the ovary. The majority of the NTP303 transcripts were localized in the vegetative cell, and were predominantly present in the apex of the pollen tube. To study the mechanism of regulation, the genomic clone NTPg303 was isolated and characterized. NTPg303 belongs to a gene family of at least five members. No introns are present in its coding region. Regions matching the pollen-specific PB-element TGTGGTT (Twell et al. 1991) have been found.

Key words Confocal laser scanning microscopy • In situ hybridization • Nicotiana tabacum Pollen-specific gene • Promoter analysis

Introduction

In flowering plants, the male gametophyte has been reduced to a cell structure of two or three cells and represents an autonomous generation of the plant's life cycle. During its short lifetime it has to perform a number of specialized functions like germination, pollen tube growth, and delivery of the sperm cells to accomplish fertilization. This imposes specific demands on the structure and organization of this organism. These specific properties have stimulated pollen research through all disciplines of biology (Giles and Prakash 1987; Blackmore and Knox 1990; Bedinger 1992; Ottaviano et al. 1992), including molecular biology (Mascarenhas 1990; McCormick et al. 1991; Scott et al. 1991b; Weterings et al. 1992).

The study of pollen development is of considerable agronomic importance for plant breeding, propagation, and seed production. At the same time, it is also an intriguing as well as a convenient model system to study the mechanism of gene regulation. The pollen genome is haploid and is actively transcribed. Specific transcripts in the microspore and pollen grain appear and disappear during its synchronous development (Schrauwen et al. 1990; Scott et al. 1991a). In the mature pollen grain, the vegetative cell and the generative cell or its derivatives, the sperm cells, have their own specialized functions. Clearly the delegation of tasks to the different cells of the male gametophyte must implicate a cell-specific regulation of genes. These properties, together with the relative ease of isolation, make the pollen grain a very suitable object for molecular biological studies.

We have set out to study gene regulation during pollen development by isolating a pollen-specific cDNA clone -NTPc303- from Nicotiana tabacum (Weterings et al. 1992). NTP303 RNA is not detectable at the mid-bi-nucleate stage of pollen development and persisted even after the pollen tube had reached the ovary. The majority of the NTP303 transcripts were detectable at the bi-nucleate stage of pollen development and can still be detected 20 h after germination and pollen tube growth in vitro. The NTP303 gene is actively transcribed during pollen tube growth. The nucleotide sequence and the derived amino acid sequence of NTPc303 show similarity to Bp10, a pollen-specific gene from Brassica napus (Albani et al. 1992). Both share similarity with members of the blue copper oxidase family. Identity between the putative NTP303 protein and these oxidases is low in the highly conserved copper binding regions. Therefore, the function or activity of NTP303 is still unknown (Albani et al. 1992; Weterings et al. 1992).

The aims of the research presented in this paper were twofold. Firstly, we wanted to further the insight into
the cell-specificity and the possible function of NTP303 in the pollen grain. Therefore, the localization of NTP303 transcripts was determined by in situ hybridization in combination with confocal laser scanning microscope (CLSM) analysis. Secondly, we aimed to identify sequences which might be responsible for the observed pollen-specific regulation. For this purpose, the sequence characteristics of the genomic clone NTPg303 were studied and its 5' region was compared to the promoter regions of other pollen-specific genes. Furthermore, the NTP303 promoter region has been searched for matches with pollen-specific cis-acting elements.

Materials and methods

**Plant material**

Plants of *N. tabacum* L. cv. Petit Havana and Samsun, *N. sylvestris*, and *N. tomentosiformis* were grown on soil under greenhouse conditions.

In situ hybridization

In situ hybridizations were carried out mainly as described by Reijnen et al. (1991). Briefly, specimens were fixed [2% glutaraldehyde/paraformaldehyde (w/v) in 60 mM phosphate buffer (pH 7.0) containing 25 mM mannitol] at 24 h room temperature. After dehydration and embedding in Paraplast, 10 μm sections were made. Sections were subsequently deparaffinized, hydrated, and hybridized to 32P-labelled anti-sense NTPc303 RNA for 16 h at 45°C in hybridization buffer [50% formamide, 0.3 M NaCl, 1 mM Tris (pH 7.5), 0.1 mM EDTA, 0.5 M Tris (pH 7.5), 0.1 mM EDTA, 1× Denhardt’s (0.02% Ficoll, BSA and PVP), 10% dextran sulphate, 60 mM DTT, 1 unit RNasin (Promega), 150 ng/μl tRNA, 300 ng/μl poly-A+ RNA]. Hybridization to 32P-labelled sense NTPc303 RNA served as a negative control. Sections were washed at room temperature to a stringency of 0.5× SSC containing 1 mM DTT, dehydrated and coated with light-sensitive emulsion (Ilford L4). After 21 days of exposure, the emulsion was developed and the nuclei in the specimens were stained with 125 ng/ml ethidium bromide. The results of the in situ hybridization were analyzed by CLSM (Biorad MRC 600).

Isolation of genomic clone

Plaque filters of a *N. tabacum* cv. Samsun genomic library constructed in λCharon 35 (Loenen and Blattner 1983) were hybridized to 32P-labelled NTPc303 according to standard procedures (Sambrook et al. 1989). A 2.9 kb SalI/EcoRI restriction fragment from the genomic clone λNTPg303, hybridizing to NTPc303, was sub-cloned into pBluescript-KS+ thus generating pNTPg303.

Plant DNA isolation and analysis

DNA was isolated according to Bernatzky and Tanksley (1986). Ten micrograms of DNA was digested with restriction endonucleases, size fractionated on an 0.8% agarose gel, and transferred to hybrid-N (Sambrook et al. 1989). The blot was hybridized to a 32P-labelled NTPc303 DNA probe for 16 h at 61°C (5× SSC, 0.5% SDS, 100 μg/ml denatured hermaphroditic sperm DNA and 5× Denhardt’s) and subsequently washed to a stringency of 0.4× SSC; 0.1% SDS at 61°C. The hybridization signal was visualized by autoradiography (3 days, −80°C, with intensifying screens).

cDNA library screening and analysis

Construction and screening of the cDNA library have been described earlier (Weterings et al. 1992). Ten thousand clones (10%) of the cDNA library were screened on plaque filters with 32P-labelled NTPc303 DNA according to standard procedures (Sambrook et al. 1989). The DNA of the resulting positive phages was analyzed by digestion with restriction endonucleases. Prior to fractionation on an agarose gel, the 3' -recessed ends were filled in by Klenow enzyme and dNTPs/25P-dATP (Sambrook et al. 1989). The bands were visualized by autoradiography (6 h, room temperature) after the gel was dried on a slab gel dryer.

**Primer extension analysis**

Primer extension analysis was performed as described (Sambrook et al. 1989). Ten micrograms of total RNA from pollen of *N. tabacum* cv. Samsun was hybridized to the 32P-labelled 30-mer 5'-TTATTTTTTTACTCTACAAAGAAGAAGG-3' (cf. Fig. 3) for 16 h at 48°C in hybridization buffer [80% formamide, 40 mM PIPES (pH 6.4), 0.4 M NaCl, 1 mM EDTA]. After precipitation, the dry pellet was dissolved in RT-mix and 200 U M-MuLV-RT was added. After reverse transcription, the RNA was treated with RNase-A. The final products were loaded on an 8% sequencing gel next to the sequence reaction produced by the same primer using pNTPg303 as template. The resulting gel was exposed for 3 days, at −80°C with intensifying screen.

DNA sequence analysis

DNA sequencing was performed by the dideoxy chain termination method (Sanger et al. 1977) using Taq DNA polymerase (Promega). Overlapping subclones were generated using exonuclease III (Henikoff 1984). Both strands of NTPg303 were sequenced using single-stranded template prepared as described by Sambrook et al. (1989). Computer analysis of the DNA sequence was performed using GCG and Intelligenetics sequence analysis software (Devereux et al. 1984).

**Results**

In situ localization of NTP303 transcripts

The exact timing and localization of expression of the NTP303 gene was determined by means of in situ hybridization and CLSM (Reijnen et al. 1991). Figure 1 shows sections of developing pollen grains and tubes. The samples were hybridized to 3H-labelled anti-sense NTP303 RNA (Fig. 1a–d, g, h). Hybridization with labelled sense NTP303 RNA (Fig. 1a–d, g, h) shows sections of developing pollen grains and tubes. The fluorescence

Fig. 1a–i In situ localization of NTP303 transcripts. Sections of microspores and pollen at different stages of development or germination. a–h Merge of fluorescence and reflection CLSM images; i dark-field image of h. a–d, g, h Anti-sense hybridization; e, f sense hybridization. a–f Arrow indicates generative nucleus; g–i Long straight arrow indicates growth direction of pollen tube. N nucleus of cell transmitting tissue, S sperm nucleus, E embryo sac. Developmental stages: a uninucleate pollen; b early binucleate pollen; c, e mid-binucleate pollen; d, f mature pollen grains; g pollen tube tip in style 24 h after germination; h, i pollen tube tip in ovary 72 h after germination.
1250 bp EcoRI/HindIII fragment from NTP303

to determine the complexity of this gene family.

To identify if NTP303 belongs to a gene family, a

expected that NTP303 belonged to a gene family.

respective restriction patterns. For these reasons, it was

shown in Fig. 2A, these clones differed in their
cDNA clones were able to identify four novel homologous ClnA

clones from the cDNA library with the cDNA clone NTP303, we

construction of (Goodspeed et al., 1994) By sequencing the pol-

result of the fusion of the genomes of N. slyferri and N.

N. lugens has an amphibiploid genome. It is the

PTP303.

PTP303.

product of PTP303, this product was

amplified by reverse transcriptase (RT) reaction (Fig. 2A).

primers were designed based on the coding region of PTP303. A

cDNA library of N. lugens was isolated from a germinating

TP303 transcripts was detected at the tip of the pollen-

TP303 transcripts were detected in the nuclei of dark-field images (Fig. 1B). The majority of the NTP303

TP303 transcripts were still detectable 20 h after ger-

TP303 transcripts were still detectable at 192 h after ger-

Previous northern blot analysis revealed that

the silver grains increased indicating the accumulation

were detected above the background. The NTP303

were shown in Fig. 1E, the first transcripts of NTP303

were detected at the mid-bicellular stage of pollen develop-

Fig. 1A shows that no transcripts could be de-

mated. Figure 1B shows the dark-field image of Fig. 1A

and reflection images obtained through CLSM were

Figs 2A-D Complexes of the NTP303 gene family. A

Resistances to the pollen tube. A map of a part of NTP303 and members of the NTP303 cDNA
(Fig. 2d) was probed to a Southern blot containing EcoRI-digested DNA from *N. tabacum*, *N. tomentosiformis* and *N. sylvestris*. The resulting blot is shown in Fig. 2b. It appeared that in *N. tabacum* NTPg303 belonged to a gene family of at least five members. Two members (8.8 and 4.4 kb fragments) seemed to be single copy genes (cf. Fig. 2c) homologous to NTPc303. The other members probably had a less extensive homology.

Although most hybridizing bands in the *N. tabacum* lane corresponded to bands from either *N. sylvestris* or *N. tomentosiformis*, the overall pattern of the bands was too complex to enable exact determination of the origin of the individual genes in *N. tabacum*.

Nucleotide sequence of NTPg303

In Fig. 3 the nucleotide sequence of the promoter region and 5’ untranslated leader of the genomic clone in pNTPg303 is shown.

To determine the transcription initiation site of NTPg303, a primer extension analysis was performed as described in methods. Figure 4 shows that this resulted in three major extension products and two minor extension products. The site of transcription initiation was arbitrarily determined at 176 bp upstream of the transcription initiation site. This corresponded to the longest extension product. It thus could be inferred from the sequence data that the position of the putative TATA box lies at -31 relative to the transcription initiation site (Fig. 3).

Comparison of the coding regions of NTPg303 and of the cDNA clone NTPc303 did not reveal any introns (data not shown). The untranslated leader of NTPg303 showed two base substitutions relative to NTPc303. They are positioned at +85 and +91, relative to the transcription start site (Fig. 3). This difference between genomic and cDNA clone can be explained by the fact that NTPc303 originates from *N. tabacum* cv. Petit Havana, whereas NTPg303 has been isolated from the genomic library of cv. Samsun. Since these substitutions are positioned in the untranslated leader sequence of NTPg303, the effect of these changes on transcription and/or the gene product is probably negligible.

In order to identify possible regulatory elements, the 5’ region of NTPg303 was surveyed for similarities to previously described pollen/anther cis-acting elements (Twell et al. 1991; Vandermeer et al. 1992). Two regions
Fig. 4 Mapping of the transcription initiation site of NTP303 by primer extension analysis. Lane 1 contains the primer extension products. The four adjacent lanes labelled T, G, C, A contain the sequence reaction produced by the same primer using pNTPg303 as template. The nucleotide sequence of the sense strand of NTP303 derived from this reaction is printed to the right (• endpoint of a major extension product, ● endpoint of a minor extension product)

Discussion

In situ localization of NTP303 expression

The CLSM analysis of the in situ hybridizations of NTP303 RNA has yielded more insight into its localization and thus the possible role of this gene in pollen. NTP303 RNA appeared to be predominantly present in the vegetative cell. In the pollen tube, the NTP303 transcripts were mainly localized in the tip region, where they persisted even after the ovary had been reached (Fig. 1g, h). The tip region of the pollen tube displays a high metabolic activity mainly supporting pollen wall synthesis (Heslop-Harrison 1987). Interestingly, recent reports on spatial organization of mRNA within animal cells have shown that intracellular activities come about by targeting mRNAs to those regions of the cell where their cognate proteins are needed (Bassell 1993; Singer 1993). Taken together, this means that, with respect to the intracellular localization and persistence of the NTP303 transcripts, NTP303 probably functions to support pollen tube growth.

Analysis of the genomic clone NTPg303

In N. tabacum NTP303 appears to be member of a gene family (Fig. 2b). This observation raises the possibility that one or more of them may be inactive pseudogenes. The fact that besides a gene family of at least five members, a cDNA family has also been identified for NTP303 (Fig. 2a), suggests that at least some members of this family are actively transcribed during pollen maturation and thus possess a functional promoter.

Since N. tabacum is the result of fusion of the genomes of N. sylvestris and N. tomentosiformis (Goodspeed 1954), it is to be expected that members of the NTP303 gene family have been contributed by both ancestors. The fact that it was impossible to determine the exact origin of the individual genes in the N. tabacum genome (Fig. 2b) was not unexpected, because it has been shown previously that the respective genomes have diverged considerably from each other (Okamura and Goldberg 1985; Buuren et al. 1992).

The identification of PB elements in the 5′ region of NTPg303 (Fig. 3) might indicate the involvement of these elements in a conserved regulatory mechanism of pollen-specific genes. Besides being identified in pollen-specific genes from L. esculentum [LAT52 and LAT56 (Twel et al. 1991)] PB elements have also been found in the promoter regions of the pollen-specific genes Zm13 in Z. mays (Hamilton et al. 1992) and the gene for α-tubulin-1 in A. thaliana [TUA1 (Carpenter et al. 1992)]. In NTP303, however, the PB elements are not in close proximity to the transcription initiation site (−253 to −513 and −328 to −322), whereas in the other genes these elements all are within −150 bp from this site. In all anther- and pollen-specific promoters analyzed, the elements conferring specificity reside within the proximity of the transcription initiation site (McCormick et al. 1991; Scott et al. 1991b). It is likely, therefore, that other elements, more proximal to the transcription initiation site, might play a role in the transcription regulation of NTP303. The region matcing the GT-2 element is positioned at −71 to −63. The GT-2 trans-acting factor has two separate binding domains; one can bind the GT-2 motif and the other has affinity for the GT-1 motif.

Computer comparisons of the NTPg303 5′ region with other promoter regions conferring pollen/anther-specificity including the α-tubulin-1 gene from Arabidopsis thaliana [TUA1 (Carpenter et al. 1992)] and Zmg13 from Zea mays (Hamilton et al. 1992) did not produce additional identities. Computer comparisons of the 399 bp promoter region of Bp10 (Albani et al. 1992) with the 5′ region of NTPg303 also did not reveal any matching regions.

showing identity with the PB core motif from LAT52 in Lycopersicon esculentum (TGTGGTT) (Twel et al. 1991) are located at positions −521 to −513 (one mismatch) and −328 to −322 (100% match). Moreover, the region surrounding the latter (−333 to −317) showed high similarity (13/14) to PBIII [TTTGTGTG-GTTAAT (Twel et al. 1991)]. Also, this region displayed 100% similarity to the binding site for GT-1 (TCTTGTGTGGTTAATAT). GT-1 is a trans-acting factor activating transcription of the pea ribulose bisphosphate carboxylase small subunit 3A gene (rbcS-3A) (Gilmartin et al. 1992). A region similar (8/9) to the binding site of GT-2 (GCGGTAATT) was identified at positions −71 to −63. GT-2 is a trans-acting factor involved in the regulation of the phytochrome A gene in rice (Dehesh et al. 1990, 1992).

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Dehesh et al. 1990, 1992). Both GT-1 and GT-2 motifs are present in the NTPg303 promoter, therefore a trans-acting factor similar to the GT-2 factor might be involved in regulating this promoter.

The expression of genes related to NTP303 in pollen of various plant species (Weterings et al. 1992) suggested a conserved regulatory mechanism. The isolation of Bp10, a pollen-specific gene from B. napus with similarities in the coding region and the pattern of expression (Albani et al. 1992), created the opportunity to compare its promoter region to that of NTPg303. Surprisingly, however, no regions matching both promoters could be detected, suggesting different regulatory mechanisms for N. tabacum and B. napus.

In conclusion, the transcription of NTP303 in the vegetative cell (Fig. 1) requires a very precise mechanism for the exact mechanism of regulation awaits an in depth analysis of the NTPg303 promoter. To this end, the promoter region of the NTP303 gene will be dissected using the microprojectile-mediated transient expression assay in pollen (Twell et al. 1989).

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The nucleotide sequence data reported has been entered in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X69440.

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