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Cellular localization of a pollen-specific mRNA by in situ hybridization and confocal laser scanning microscopy

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Summary. The application of confocal laser scanning microscopy together with in situ hybridization experiments in tobacco pollen enabled a detailed localization of a pollen-specific mRNA. The three-dimensional distribution of this specific mRNA over the whole pollen grain was reconstructed by means of optical sections of one specimen.

Key words: In situ hybridization – Pollen-specific gene expression – mRNA – Confocal laser scanning microscopy

Introduction

When a plant reaches maturity a set of reproductive organs and cells are generated that contain specialized tissue types such as pollen, style and petals (Buchen and Sievers 1981; Giles and Prakash 1987; Mascarenhas 1990). The reproductive organs of plants are made up of tissues that are functionally and morphologically distinct from one another as a consequence of differential gene expression and the formation of tissue-specific mRNAs (Budelier et al. 1990; Clark et al. 1990; Goldberg et al. 1989; Kuhlemeier et al. 1987; Mariani et al. 1990; Mascarenhas 1988, 1990). In lily and tobacco differences have been observed in the accumulation of mRNAs in various developmental stages of the microspores and microgametophytes (Schrauwen et al. 1990): some mRNAs accumulate transiently, while others arise during the last phases of pollen development and remain stable in mature pollen. Such results show that pollen development is accompanied by the formation of specific mRNAs that may function either at certain moments in development or during the fertilization process. Though the functional properties of such mRNAs have not been demonstrated yet, their occurrence indicates their involvement in these processes. Precise localization of these specific RNAs may provide further information about the functions of these RNAs in the pollen.

Localization can be carried out by in situ hybridization, but its suitability is dependent on the efficiency of the detection system (McFadden 1989). The small size of the pollen grain (30 μm) harbouring the sickle-spindled generative cell (5 × 20 μm) requires an optimal detection of the hybridization signals.

Confocal laser scanning microscopy (CLSM) provides a high resolution due to the detection of the in-focus field only. Stepwise decrements in the depth of field gives rise to optical sectioning in the Z-direction and the registration of the reflection of individual hybridization signals, which results in a high resolution (Amos et al. 1987; Harders et al. 1989; Houtsmuller et al. 1990; Shotton 1989). The application of in situ hybridization techniques in combination with CLSM provides a unique possibility to localize specific mRNAs particularly in such a tiny plant organ. This report is the first of its kind in which the in situ detection of a specific plant mRNA is defined by confocal laser scanning microscopy.

Materials and methods

Mature pollen grains from *Nicotiana tabacum* L. 'Petit Havanna' SR1 (Maliga et al. 1973) were collected at anthesis as described earlier by Herpen et al. (1989) and prepared for in situ hybridization. A procedure was followed that varied from that used with other plant tissues (Anderson et al. 1986; Angerer et al. 1987; Hanson et al. 1989; McFadden 1989). Due to the presence of the pollen wall, the pollen grains were washed 3 times with MP buffer (100 m*M* mannitol, 58 m*M* KH_2PO_4, pH 7.0), fixed for $2^1/_2$ h at room temperature in GPMP buffer (2% glutaraldehyde and 2% paraformaldehyde in MP) and washed once again 3 times with MP buffer. The pollen suspensions (final concentrations 2×10^6 grains per ml) were mixed with molten low-melting agarose (final concentration 0.5%), solidified at 4° C and cut into small pieces.

This material was dehydrated by passage through an alcohol series (5%, 10%, then 10% steps up to 90%, 20 min each), followed by alcohol (95% and 100%, for 30 min) and an alcohol-xylene series (25%, 50% and 75%, 30 min) and 100% (16 h). The prepara-

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tions were then passed into paraplast: 4 h in 5% paraplast at 25° C; 4 h in 10% at 30° C; 2 h in 25%, 37% and 50% at 42° C; 2 h in 67% and 75% at 58° C; 72 h in 100% under vacuum at 58° C. From these blocks 10-µm-thick sections were cut and mounted on slides (Angerer et al. 1987).

The mounted slides were deparaffinized, hydrated and prehybridized according to Cox et al. (1984) except that the proteinase K treatment was followed by a series of brief washings with water, 100 m*M* ethanolamine, pH 8.0, and 100 m*M* ethanolamine: acetic anhydride (v/v = 4/1), and again dehydrated.

Hybridization and post-treatment were carried out as described by Hanson et al. (1989) with minor modifications as the replacement of RNA by 150 µg/ml^{-1} tRNA, 350 µg/ml^{-1} polyA-RNA and 40 µ/ml^{-1} RNAsine in a total volume of 30 µl per slide.

The labeled probes had an activity of 2×10^5 cpm. The probes used in the in situ hybridization were a [35-S]- or [3-H]-labeled antisense and sense RNA with lengths of 460 and 320 nt respectively for the [35-S] probes and 180 and 320 nt respectively for the [3-H] probes. The probes were synthesized by in vitro transcription of a pollen-specific cDNA clone (pNTP303) subcloned in the pBluescript II KS(+) vector (Stratagene). This pollen-specific cDNA clone with a length of 2 kbp was obtained from a cDNA library prepared against mRNA from mature tobacco pollen. The library was differentially screened against mRNA of various tissues of the plant and the flower.

The samples were immersed carefully in the light-sensitive emulsion (Ilford K2 and L4) so that it penetrated into the pollen tissue. After an exposure time of 10 days ([35-S] probes) or 20 days ([3-H] probes) at 4° C the developed silver grains were detected in the emulsion by measuring the reflectance with the BioRad MRC-500 confocal laser scanning microscope. The position of the pin-hole was set in such a way that overlap between the adjacent layers at 1 µm distance was only slight.

Results

The applied fixation procedure resulted in pollen grains that were smooth, without invaginations, and oval in shape (Fig. 1). Hybridization signals present as silver grains in the specimen can be measured by reflection when observed with CLSM and by absorption when observed with normal microscopy. Analyses of the hybrid-

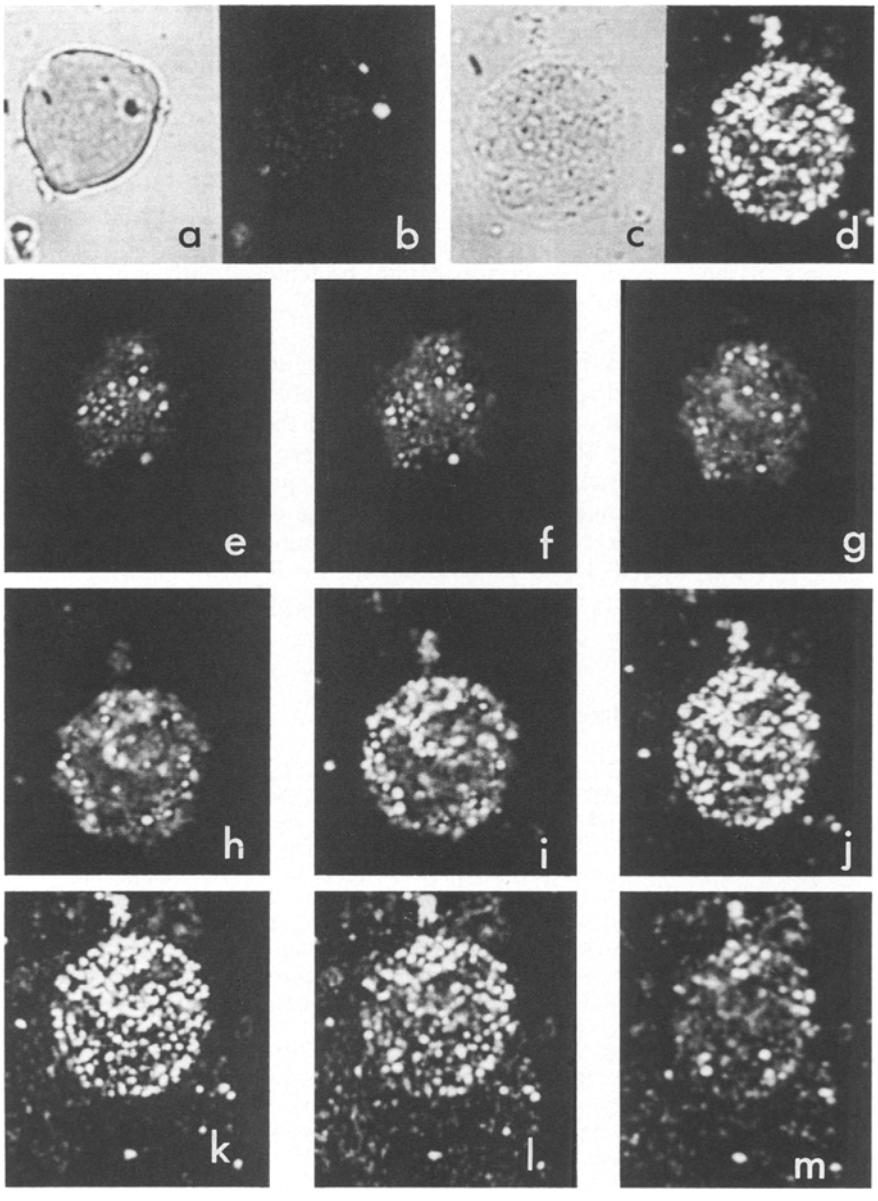

Fig. 1a–m. Localization of a pollen-specific NTP303 mRNA in pollen by non-confocal transmission (**a, c**) and confocal laser scanning (**b, d–m**) microscopy. **a, b** Pollen hybridized using sense RNA; **c, d** pollen hybridized with antisense RNA. **e–m** Represent one specimen which is optically divided in nine distinct focus levels at 1 µm distances. *Small dark spots* (developed silver grains) in a and c represent regions of RNA/RNA hybridization; *bright white spots* in b and d–m are reflections of developed silver grains and represent regions of RNA/RNA hybridization. Probes were labeled with [35-S]UTP

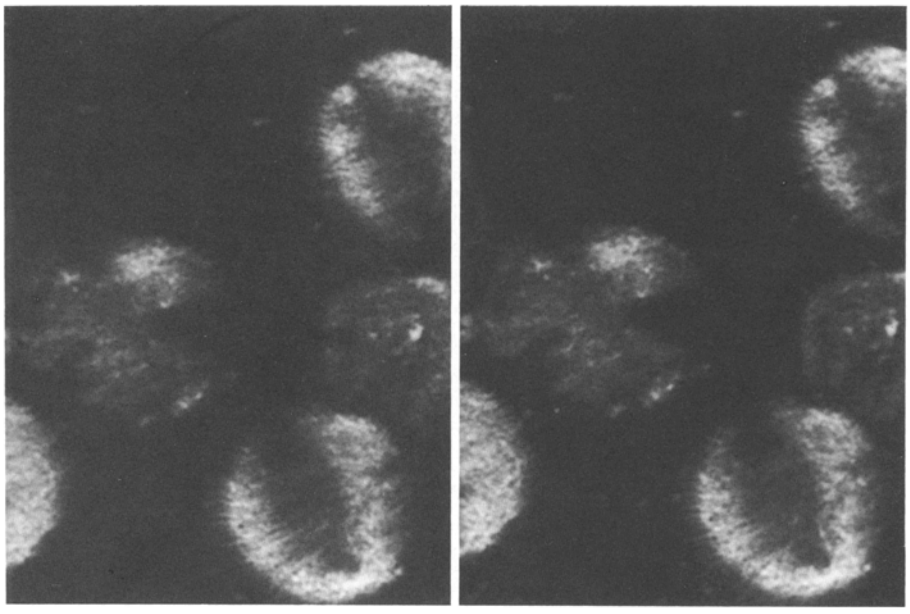

Fig. 2. A stereo-projection image obtained after digital reconstruction of eight individual optical sections in the z-direction of one specimen. Hybridization signals were achieved after application of the antisense probe labeled with [3-H]UTP

ization with the antisense RNA probe (pNTP303) in this way caused a more pronounced signal with CLSM (Fig. 1 d) than with normal microscopy (Fig. 1 b). When the sense probe was used neither reflection (Fig. 1 b) nor absorption (Fig. 1 a) could be observed.

The localization of this pollen-specific mRNA with the antisense probe in combination with CLSM visualized the distribution of this RNA as tiny spots in the cytoplasm of the vegetative cell (Fig. 1 k). This distribution of the antisense hybridization signals in the whole specimen obtained with CLSM (Fig. 1 e–m) means that the emulsion was penetrated completely in the pollen grain. Optical sectioning at 1 μm distances of the antisense-treated pollen with CLSM showed that the accumulation of this RNA is uniform for the different optical layers of the 10-μm-thick section (Fig. 1 e–m). So measurement of the reflection in the z-direction delivered a three-dimensional distribution of the specific RNA as shown in Fig. 2 for the distribution of NTP303 in one specimen of a few pollen grains from tobacco. This was achieved by a reconstruction of eight optical layers (at 1 μm distances) of one specimen in the z-direction with the CLSM software. The application of this technique results in a stereo-projection image in which the distribution of the pollen-specific RNA is shown.

Discussion

The application of in situ hybridization together with CLSM resulted in a detection of the hybridization signal in tobacco pollen with clear contrast and precise localization. By this method a 10-μm-thick specimen can be optically divided in the z-direction into sections that are at a 1 μm distance from each other (Houtsmuller et al. 1990). Optical sectioning of a specimen offers the possibility to detect the location of mRNAs in all three directions of the specimen. The CLSM technique is also less labourious and distortive than the conventional method consisting of semi-thin serial sectioning and non-confocal microscopy (Pawley 1990; Shotton 1989). Under the conditions of the experiments performed, optical sections at 1-μm distances were in focus. The distribution patterns of the silver grains of adjacent optical sections of the same specimen were different (Fig. 1 e–g). In non-confocal microscopy, e.g. transmission light microscopy, optical sectioning cannot be achieved with semi-thin sections (10 μm) due to the interference of out-of-focus regions in the in-focus field.

With CLSM a stereo-projection image of the hybridized spots could be achieved after electronic reconstruction of the optical sections of the specimen. Pollen-specific RNA NTP303 was observed to be distributed over the whole pollengrain as tiny spots (Figs. 1 and 2). This manner of distribution and the pollen specificity suggest that NTP303 has a special function in the vegetative cell. De novo synthesis of this RNA in growing pollen tubes (Weterings et al. 1991) supports this hypothesis.

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