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TECHNICAL ADVANCE

Specific distribution of mRNAs in maize growing pollen tubes observed by whole-mount *in situ* hybridization with non-radioactive probes

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Summary

The distribution of a number of specific mRNAs has been observed in maize growing pollen tubes. Whole-mount *in situ* hybridization using digoxygenin-labelled RNA probes has been tested. The technique appears to be a simple, rapid and reliable method in this system, in immature anthers and also in other tissues. Results for three probes are presented. They correspond to a hydroxyproline-rich glycoprotein (HRGP), an abundant component of the maize cell wall, to an α-tubulin (encoded by the *Tubα 1* gene) highly expressed in the radicular system of the plant and also in pollen, and to an isoform of the malic enzyme, involved in the basic metabolism of the plant. The mRNAs corresponding to these three proteins are differently distributed in the germinating pollen. While HRGP mRNA is only present in the tube, malic enzyme mRNA is only present in the body of the pollen cell, and α-tubulin mRNA is present in both parts of the cell but shows a higher accumulation in the tip of the pollen tube.

Introduction

In flowering plants, the male gametophyte is reduced to a single cell, the pollen grain. Pollen is a special plant cell type where a high number of genes are expressed (Willing *et al.*, 1988). This high number corresponds to the large number of functions required for the biosynthesis of pollen, for the maintenance of the latent state of the pollen grain and for the metabolism of the germinating cell. In most of the plants studied, the mRNAs or proteins required for germination are already present in the mature pollen grain, and new protein synthesis is required only for growth of the pollen tubes following germination. Although the pollen of most plants studied contains ribosomes, mRNAs and tRNAs, new RNA synthesis does occur during pollen germination and tube growth (Mascarenhas, 1988; Ursin *et al.*, 1989). The nature of pollen tube growth is different from other plant cell types. Pollen tube growth is restricted to the tip region, and the rapidity of the growth requires a flow of products towards the growing end (Mascharenhas, 1993).

During the formation of the pollen tube a number of elements have been shown to be essential: (i) the elements needed for the biosynthesis of the expanding cell wall in the tip of the tube (Mascarenhas, 1993); (ii) microfilament and microtubule components that have been shown to be abundant in the growing tube (Pierson and Cresti, 1992; Raudaskoski *et al.*, 1987); (iii) biosynthetic enzymes required for the general metabolism of the pollen cell.

A technique widely used in animal systems, especially in *Drosophila* embryos, is whole-mount *in situ* hybridization (Tautz and Pfeifle, 1989). Similar techniques have been employed also in plant systems using antibodies (Ludevid *et al.*, 1992) or ribosomal RNA probes (Bauwens *et al.*, 1994). This technique has been applied here to germinating pollen and immature anthers. The method, coupled with the use of non-radioactive probes provides a rapid and simple way to examine the mRNA accumulation preserving the basic organization of the tissues with minimal disruption.

In the present report, the accumulation of mRNA corresponding to three different proteins has been analysed in the developing maize pollen tube. The three probes correspond to the hydroxyproline-rich glycoprotein (HRGP), one of the most abundant components of the maize cell wall (Kieliszewski *et al.*, 1990; Stiefel *et al.*, 1988, 1990); an α-tubulin, a component of the cytoskeleton whose gene (*Tubα 1*) has been shown to be highly expressed in meristematic tissues, but preferentially in the radicular system and in pollen of maize (Montoliu *et al.*, 1989, 1990); and a NADP-dependent malic enzyme isoform (Rothermel and Nelson, 1989), an enzyme that is involved in different metabolic pathways in the maize plant. In this report we show that the different mRNAs have a defined and distinct distribution inside the germinating pollen cell, and that the technique of whole-mount *in situ* hybridization can be applied to other plant tissues.

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Results and Discussion

Localization of HRGP mRNA in growing pollen tubes

When a HRGP riboprobe is used (Stiefel *et al.*, 1988), mRNA is observed in the pollen grain but mainly in the germination aperture (Figure 1a). The specific concentration of the mRNA in the growing tube is more evident as growth progresses (Figure 1b). It is then possible to observe that the tube is heavily stained while the body of the well is not. This high concentration of mRNA corresponding to one of the main elements of the maize cell wall may be in accordance with the need for cell wall components in the rapidly growing tube. Although there have been a number of morphological studies on pollen wall development (Mascarenhas, 1993), biochemical studies of this highly specialized pollen structure are only just starting. Maize HRGP mRNA has been shown to be present at early stages of the cell wall formation in actively dividing cells (Ludevid *et al.*, 1990) and especially in the developing embryo (Ruiz-Avila *et al.*, 1992). In the case of the growing pollen tube the same component appears to be needed where cell wall is being formed in the absence of cell division.

Localization of an α-tubulin mRNA in germinating pollen grains

Microfilaments and microtubules are structures abundantly present during pollen germination which have been shown to play a key role in this phenomenon. When an α-tubulin probe is used, the pattern of mRNA distribution is different (Figure 1e, f and g) to the one observed for the HRGP probe. The probe corresponds to the *Tubα 1* gene which is expressed in the meristematic tissues of maize, preferentially in the root meristem, and is highly expressed in pollen (Montoliu *et al.*, 1989). It has also been shown that in tobacco transgenic plants the promoter of gene *Tubα 1* is functional from the first meiotic division and in the mature pollen (Rigau *et al.*, 1993). When the α1-tubulin probe is used in whole-mount *in situ* hybridization of germinating pollen, the mRNA is distributed both in the cell and in the tube. However, it is possible to observe that the distribution within the tube is not regular and a particularly high level of accumulation can be observed in the tip of the tube.

Localization of a malic enzyme mRNA in the body of the pollen grain

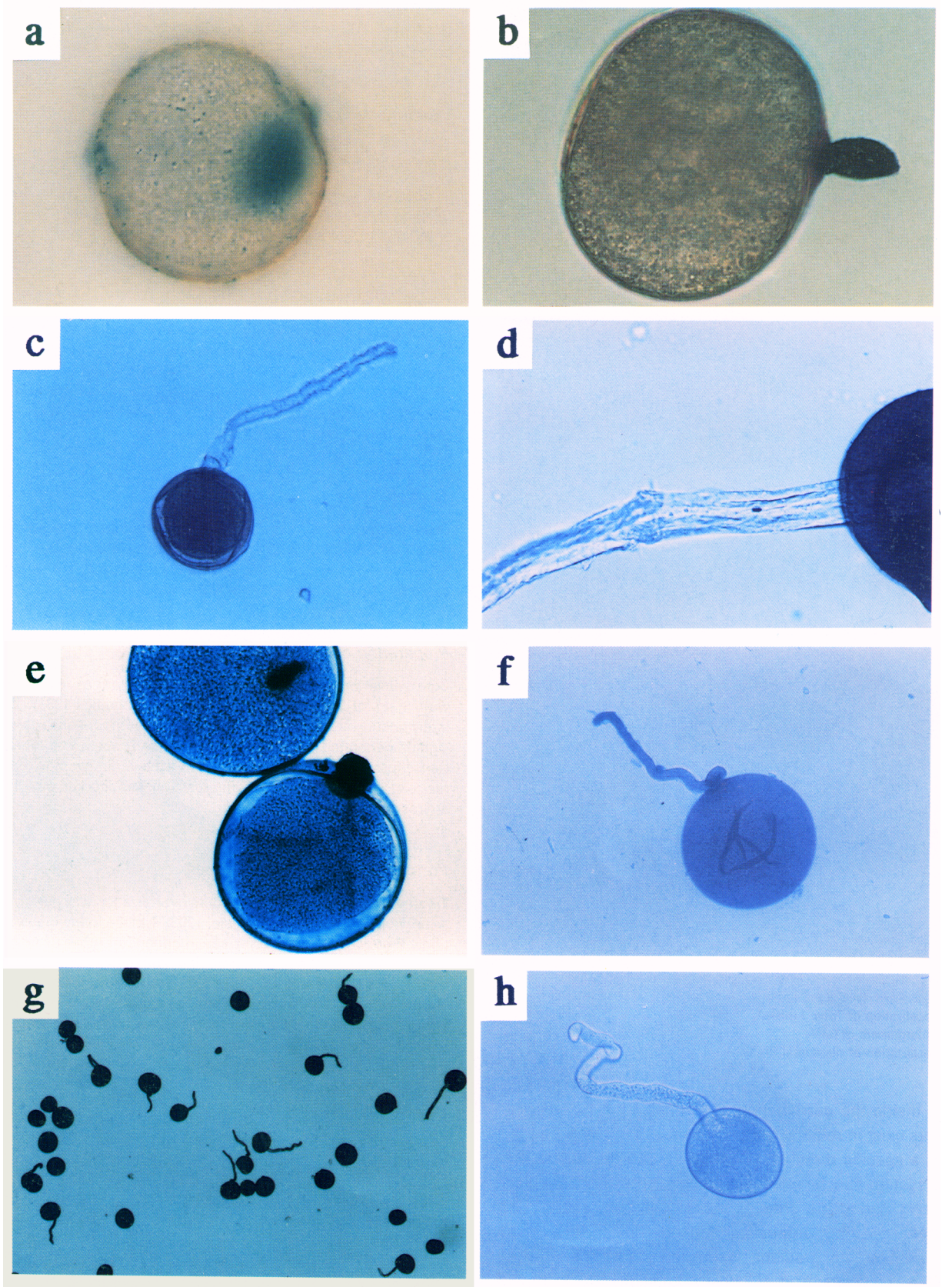
Malic enzymes play an essential role in different metabolic pathways (Rothermel and Nelson, 1989). In contrast to the two above-mentioned probes, when a probe corresponding to the NADP-dependent malic enzyme (Rothermel and Nelson, 1989) is used, specific mRNA is only observed in the body of the pollen cell and not in the germinating pollen tube (Figure 1c and d).

Whole-mount in situ hybridization in plant tissues

The technique of whole-mount *in situ* hybridization can also be used in other tissues; it has been successfully used in young organs such as roots, shoots or leaves (data not shown). As an example, the same hybridization carried out in male inflorescences is shown (Figure 2). In this figure inflorescences were hybridized with an α-tubulin probe corresponding to the *Tubα 1* gene. It is possible to observe that the mRNA specifically accumulates in the immature anther and not in somatic tissues (Figure 2a and b). When a riboprobe corresponding to a ribosomal RNA gene is used, the somatic tissue of the anther is also labelled (Figure 2c) indicating that the probe readily diffuses to all the tissues. At least four independent experiments were done with each gene, and more than 95% of the individual pollen grains showed the corresponding expression pattern. Controls with sense riboprobes were done in all cases and gave no observable signal on germinating pollen grains (Figure 1h), or other tissues (data not shown).

The use of whole-mount *in situ* hybridization allows the rapid detection of specific mRNAs in different parts of the growing pollen tube. From our results it appears that mRNAs coding for structural elements of the cell wall such as HRGP, or components of the microtubules such as α-tubulin, are concentrated in the growing parts of the pollen tube. This effect is especially clear in the case of HRGP and it is easily correlated with the active building of cell wall in the growing tube. In contrast, the mRNA coding for the malic enzyme, a protein that takes part in the general metabolism of the cell, is mainly present in the body of the pollen cell. It is interesting to note that one of the probes used in the present study (tubulin α1) corresponds to a cytoskeletal protein indicating that microtubules may take part in this process. These results indicate

Figure 1. Whole-mount *in situ* hybridization to hydroxyproline-rich glycoprotein (HRGP), malic enzyme (ME) and α-tubulin (*Tubα 1*) mRNAs in germinating maize pollen grains.
The accumulation of specific mRNAs was observed *in situ* using digoxygenin-labelled riboprobes.
(a and b) Localization of HRGP mRNA in growing pollen tubes.
(c and d) Localization of the malic enzyme encoding mRNA in the body of the pollen grain.
(e, f and g) Localization of *Tubα 1* mRNA in the pollen grain and tube.
(h) A pollen grain hybridized with the *Tubα 1* sense RNA probe.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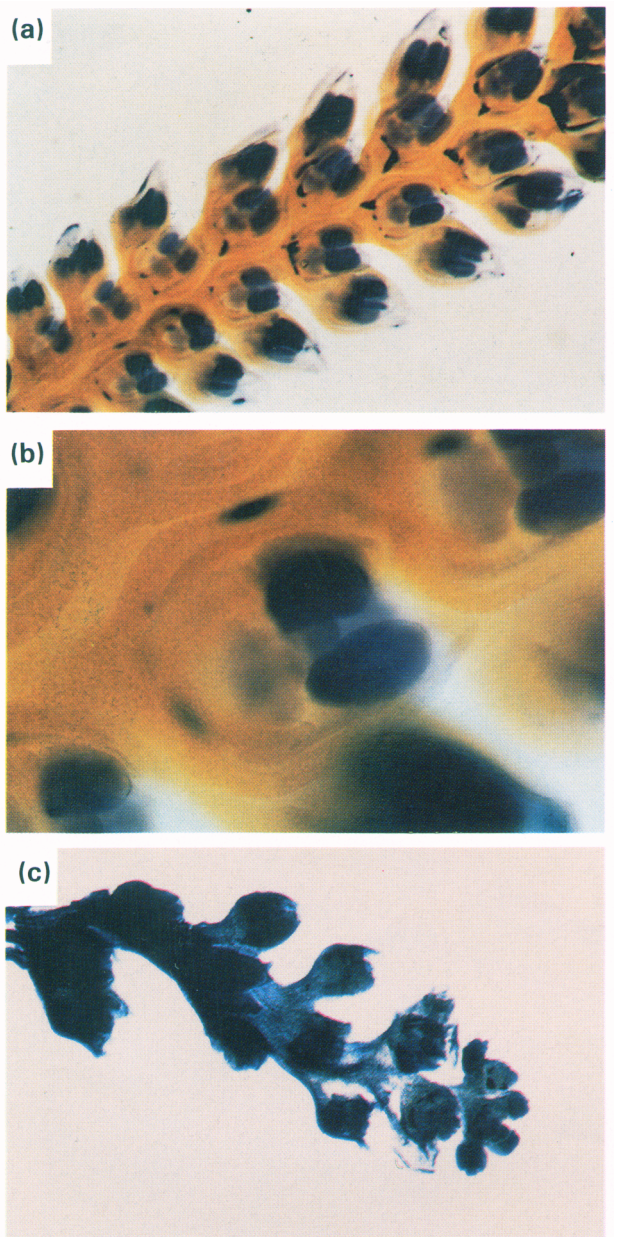

Figure 2. Whole-mount *in situ* hybridization to α-tubulin (*Tubα 1*) mRNA and rRNAs in developing anthers.
(a) Localization of *Tubα 1* mRNA.
(b) Enlargement of (a).
(c) Localization of ribosomal RNA.

that inside the germinating pollen cell, specific compartments exist in relation to the presence of mRNAs. The fact that a specific distribution of mRNA can be observed in this system may be related to mechanisms distributing cell content during the growing process. In fact, a streaming pattern carrying organelles and vesicles, and also an intracellular ion gradient, is formed inside the growing pollen cell (Pierson *et al.*, 1994). Consequently, a flow of specific materials including mRNAs is probably produced within the cell, towards the location where the gene product is needed. Whole-mount *in situ* hybridization appears to be an appropriate technique to study such a phenomenon.

Experimental procedures

Plant material and growth conditions

Experiments were carried out with an inbred line of *Zea mays* (Pioneer B73). Seeds were grown in the greenhouse in Barcelona in soil at 22°C with a 16 h light/8 h dark regime.

Pollen tube germination

Pollen grains were collected from freshly opened flowers and spread directly into a liquid culture medium. The medium contained 12% sucrose, 0.03% $CaCl_2$, 0.01% H_2BO_3 and 0.2% DMSO (Raudaskoski *et al.*, 1987; Walden, 1993). The pollen grains were incubated in darkness for 1 h at 20°C. Most germination took place within 30 min.

Fixation

Developing anthers, mature pollen, or germinated pollen grains were fixed in 70%:20%:10%, ethanol:acetic acid:DMSO for 1 h at room temperature. Once the fixative was removed, the samples could be stored in 70% ethanol at 4°C indefinitely.

Preparation of riboprobes

Both sense and antisense transcripts were synthesized starting with 1 μg of linearized DNA template (Langdale, 1993). Data interpretation is absolutely dependent upon comparisons between signals generated by sense and antisense probes. The labelled transcript was hydrolysed by alkali to achieve an average length range of 150–200 nt. The labelling reaction was checked on a 1.5% agarose gel (Langdale, 1993).

Pretreatment of cells and tissues

Treatment of cells and tissues prior to hybridization was performed as previously described (Langdale, 1993) with the following exceptions. Pretreatment was performed in Eppendorf tubes using 1 ml of solution each time. Rehydratation and dehydratation series were carried out for 5 min each. Pretreatment of samples was started with the 70% ethanol of the dehydration series. After the 5 min incubation in 100% ethanol the solution was removed and replaced by xylene (2×10 min). Samples were then rehydrated through an ethanol series. Incubation with 1 μg ml^{-1} of proteinase K was carried out for 40 min followed by blocking of the protease with 0.2% glycine for 5 min.

Hybridization and washes

Tissues and cells were prehybridized for 1 h at 55°C in 100 μl of hybridization solution (50% formamide, 6×SSC, 3% SDS, 100 μg ml^{-1} tRNA, 100 μg ml^{-1} poly A). Hybridization was performed in 100 μl at 55°C overnight. A probe concentration of 500 ng ml^{-1} in hybridization buffer (prewarmed at 55°C) was used. The hybridization solution was removed and the tissues or cells were rinsed

twice with 1 ml of 1×SSC, 0.1% (w/v) SDS at room temperature and washed 3×10 minutes with 1 ml of 0.2×SSC, 0.1% (w/v) SDS at 55°C. After rinsing with 1 ml of 2×SSC for 5 min at room temperature an incubation with RNase A was carried out for 1 h at 37°C in a prewarmed 10 μg ml^{-1} RNase A in 2×SSC solution. Samples were then rinsed twice in 2×SSC before proceeding to the next stage.

Blocking, antibody incubation and washes

The hybridized probes were detected using an alkaline phosphatase antibody conjugate according to suppliers' protocols (RNA colour kit for non-radioactive *in situ* hybridization, Amersham). The following series of incubations were performed with gentle shaking and at room temperature. Samples were washed with 1 ml of TBS (100 mM Tris–HCl, pH 7.5, 400 mM NaCl) for 5 min. They were then incubated in 1 ml of block solution (0.5% (w/v) blocking agent in TBS) for 1 h and rinsed in TBS for 5 min. The antibody was diluted 1:1000 in 0.5% (w/v) BSA Fraction V in TBS. The incubation with the antibody was performed in 100–200 μl for 2 h. Samples were then washed for 3×10 min in TBS.

Detection

Samples were washed in detection buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min. The detection buffer was then replaced by a fresh one containing NBT (4-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate). Forty five microliters of NBT (75 mg ml^{-1} NBT in 70% dimethylformamide) and 35 μl of BCIP (50 mg ml^{-1} in dimethylformamide) were added to 10 ml of detection buffer. The volume of detection reagent applied to each tissue was 100–200 μl. The length of time required to obtain a result depended on the gene studied, but normally an overnight incubation was required. All detection reactions were stopped after a 12 h incubation. Samples were then dehydrated, infiltrated in xylenes for 5 min and mounted in Permount.

Digoxygenin-labelled hybrids were viewed using brightfield microscopy and photographs were taken using Ektachrome 160 films.

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