BIOLOGIA PLANTARUM 43 (4): 517-522, 2000

Temporal changes in the RNA distribution between polysomes and postpolysomal ribonucleoprotein particles in tobacco male gametophyte

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Abstract

Growth of tobacco (*Nicotiana tabacum* L.) pollen tube is controlled by post-transcriptionally regulated protein synthesis. Stored mRNA was found to be present in the form of messenger ribonucleoprotein particles (mRNPs) in both maturing and germinating pollen. During pollen dehydration in the anthers content of mRNPs strongly increased during dissociation of polysomes suggesting a transfer of mRNA liberated from polysomal structures into mRNP particles. Pollen germination and initial pollen tube growth characterized by rapid reassociation of ribosomes was accompanied by decrease of mRNPs indicating an involvement of mRNA of these particles in the formation of polysomes. Distribution of the particular mRNAs during the pollen activation preceeding tube growth suggests that other mechanisms along with pollen rehydration are engaged in this process and that the continuous exploitation of stored mRNPs during pollen tube growth is precisely regulated.

Additional key words: *in vitro* translation, *Nicotiana tabacum* L., pollen, polysomes, stored mRNA, translational regulation.

Introduction

Tobacco male gametophyte is a system characterized by a high extent of post-transcriptional regulation of gene expression (Goldberg 1988, Mascarenhas 1993). This type of regulation is obviously dependent on the existence of stored, translationally inactive mRNA. Stored mRNA has repeatedly been found in the form of free messenger ribonucleoprotein particles (free mRNPs) in functionally specialized systems where development is strictly time-limited, such as seeds, ovules and embryos. The phenomenon of stored mRNPs has been studied intermittently since its discovery in animal (Spirin and Nemer 1965) and plant tissues (Silverstein 1973). Most experimental data have been obtained from animal systems by analysis of RNP particles masking stored maternal mRNA in sea urchin (Davidson 1976), goldfish (Katsu *et al.* 1997), *Xenopus laevis* (Pierandrei-Amaldi and Amaldi 1994) embryos.

In plants, the information on stored mRNA is more limited. This phenomenon was studied in dry wheat seeds (Silverstein 1973), maize germinating seeds (Beltrán-Peña *et al.* 1995, Rincón-Guzmán *et al.* 1998), *Tortula ruralis* gametophytes (Wood and Oliver 1999) and alfalfa somatic and zygotic embryos (Pramanik *et al.* 1992). The presence of stored mRNAs in mature pollen has been demonstrated in several species (Mascarenhas and Bell 1969, Linskens *et al.* 1970, Tupý 1982, Tupý *et al.* 1977, Schrauwen *et al.* 1990), but there is no evidence for their existence in the form of free mRNPs.

Our study focuses on determining a) the formation of mRNPs and the presence of stored mRNA in this form in immature tobacco pollen, b) the derepression of stored mRNA in the activation process, and c) the presence of free mRNPs as an available source of fresh mRNA during pollen tube growth.

Received 8 November 1999, *accepted* 21 March 2000.

Abbreviations: AMD - actinomycin D; DMDC - dimethyldicarbonate; DTT - dithiothreitol; MES - 2-morpholino-ethanesulfonic acid; mRNP - messenger ribonucleoprotein particle; M_r - relative molecular mass; PMSF - phenylmethylsulfonyl fluoride; PTE - polyoxyethylene-10-tridecyl ether, SDS - sodium dodecylsulphate; SDS-PAGE - polyacrylamide gel electrophoresis with SDS; TCA - trichloracetic acid.

Acknowledgments: This work was supported by the grant 204/94/0654 of the Grant Agency of the Czech Republic, the grant A5038801 of the Grant Agency of the Academy of Sciences of the Czech Republic, and by the grant VS 96145 of the Ministry of Education of the Czech Republic.

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Materials and methods

Plant material: Immature pollen grains of *Nicotiana tabacum* L. were isolated from the anthers at three stages of development; at the beginning of mitosis (stage 1), mid-bicellular stage (stage 3) and one day before anthesis (stage 5, Tupý *et al.* 1983). Pollen tubes were cultivated in SMM-MES medium under sterile conditions as a shaken suspension (Tupý and Říhová 1984).

Subcellular fractionation: Sedimentation of polysomes was performed essentially according to de Vries *et al.* (1988) with several modifications. 100 mg of pollen grains or pollen tubes corresponding to 100 mg of cultivated pollen was homogenized in sterile mortar with pestle under liquid nitrogen with 0.2 cm^3 of polysomal buffer (PB; 200 mM Tris-HCl, pH 9.0, 400 mM KCl, 60 mM Mg-acetate, 2 mM DTT, 0.5 mM PMSF, 1 % PTE, 1 mM cycloheximide, 250 mM sucrose) and transferred to centrifuge tube. 7 cm^3 PB was added, the tube was centrifuged (23 000 *g*, 10 min, 4 °C) to obtain postmitochondrial supernatant. The postmitochondrial supernatant was centrifuged (300 000 *g*, 1 h, 4 °C, *Beckman 75Ti*, *Beckman Instruments*, Palo Alto, USA) through the 1.5 cm^3 cushion of 60 % sucrose in gradient buffer (GB; 40 mM Tris-HCl, pH 8.5, 100 mM KCl, 30 mM Mg-acetate, 2 mM DTT, 0,5 mM PMSF, 1 mM cycloheximide) to sediment polysomes. The supernatant containing post-polysomal fraction was then centrifuged again (300 000 g, 16 h, 4 °C) to separate postpolysomal mRNPs by sedimentation. Total RNA was extracted from both pelleted fractions (Chomczynski and Sacchi 1987). mRNA was purified from total RNA with *PolyATract*™ mRNA Purification System (*Promega Corporation*, Madison, USA) according to manufacturer's instructions.

Sucrose gradient centrifugation: 0.2 cm^3 of postmitochondrial supernatant prepared as described above was centrifuged (100 000 *g*, 3 h, 4 °C, *Beckman NVT90*, *Beckman Instruments*, Palo Alto USA) in 10 - 40 % linear sucrose gradient in GB in a total volume of 4.8 cm^3. After centrifugation, 0.4 cm^3 fractions were collected from which total RNA was then extracted (Chomczynski and Sacchi 1987). All work with sucrose gradients except centrifugation was done on the *Econo System* (*Bio-Rad Laboratories*, Hercules, USA).

Translation *in vitro* in heterologous system and protein analysis: 10 μg of total RNA, RNA isolated from polysomes and from postpolysomal fraction was translated under *in vitro* conditions using the *Rabbit Reticulocyte Lysate System* (*Promega Corporation*, Madison, USA) according to the manufacturer's instructions. Labeled proteins were separated by one-dimensional electrophoresis on 10 % and 12.5 % polyacrylamide gels (SDS-PAGE; Laemmli 1970), fluorographed (Čapková *et al.* 1987) and evaluated densitometrically at 542 nm with *Ultroscan XL* (*Amersham Pharmacia Biotech AB*, Uppsala, Sweden).

Translation *in vitro* in semihomologous system: Polysomes were pelleted by centrifugation as described. The polysomal pellet was dissolved in 0.05 cm^3 of sterile H_2O. The amount of polysomal RNA was estimated spectrophotometrically by measuring absorbance at 260 and 280 nm. Fraction of polysomes corresponding to 10 μg of polysomal RNA was then translated under *in vitro* conditions using the *Rabbit Reticulocyte Lysate System* according to the manufacturer's instructions.

Results and discussion

Existence of free RNPs and mRNA re-distribution between free RNPs and polysomes: The post-mitochondrial supernatant was centrifuged through 10 - 40 % sucrose density gradient and three important groups of peaks, corresponding to polysomes, monosomes and postpolysomal mRNPs, were found. Microspores at the

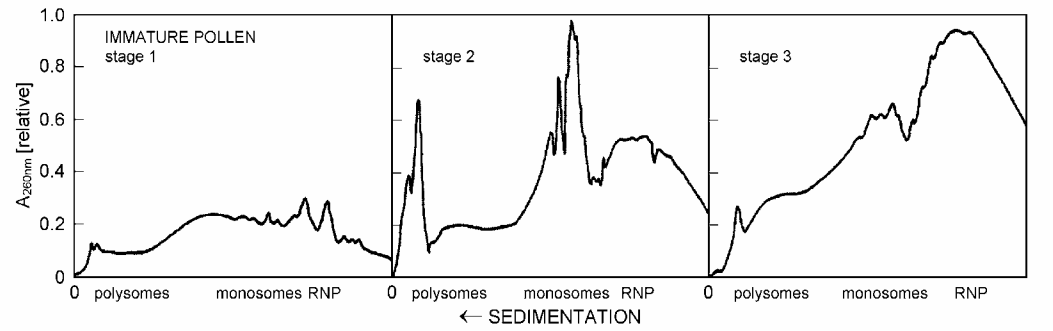

Fig. 1. Absorbance profiles of postmitochondrial pollen fraction on sucrose gradients at stages 1, 3 and 5 of pollen development. Peaks corresponding to polysomes, monosomes and postpolysomal RNPs are marked.

beginning of mitosis (stage 1) possesed only a small amount of total RNA (Fig. 1). The limited number of polysomes corresponded to low translational activity at this stage of development (Tupý *et al.* 1983). Nevertheless, the detectable amount of RNA in the postpolysomal free RNP fraction demonstrated the formation of stored mRNA at the very beginning of pollen development. Immature pollen at the initiation of starch deposition (stage 3) exhibited maximal rates of protein as well as RNA synthesis (Tupý *et al.* 1983). This was demonstrated by the occurrence of two major peaks at the positions of polysomes and monosomes. The increasing portion of postpolysomal RNPs showed the continuous synthesis of stored mRNA (Fig. 1). Decreases in transcription and translation were characteristic for later stages of pollen maturation (stage 5; Tupý *et al.* 1983) and were accompanied by re-distribution of RNA from polysomes to free RNPs (Fig. 1).

Imbibition represents the activation phase of pollen grain (Heslop-Harrison and Heslop-Harrison 1992) and precedes pollen germination and tube growth. During the first 10 min of tobacco pollen imbibition, stored mRNA associated with ribosomes into polysomal complexes to enable protein synthesis necessary for pollen tube growth (Tupý *et al.* 1977). The proportion of polysomes further increased during pollen tube growth *in vitro* and reached a maximum after 4 h of pollen cultivation. Our results confirmed the strong increase of polysomes during pollen imbibition and initial tube growth and showed that this increase was accompanied by decrease of mRNPs (Fig. 2).

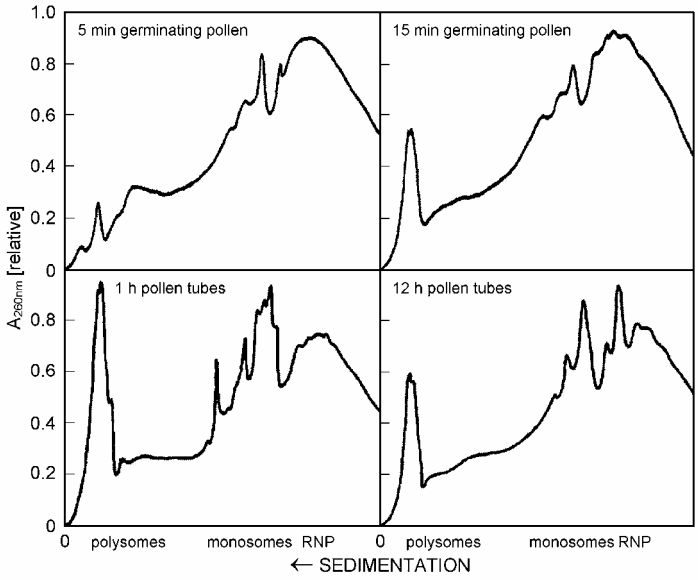

Fig. 2. Sucrose density gradient centrifugation of postmitochondrial fraction isolated from pollen grains imbibed for 5 and 15 min and from pollen tubes cultivated *in vitro* for 1 h and 12 h. Peaks corresponding to polysomes, monosomes and postpolysomal RNPs are marked.

Pollen tubes growing *in vitro* in SMM-MES medium exhibited high rates of protein synthesis for 18 h of cultivation (Čapková *et al.* 1983). After this period the gradual cessation of both translation and growth activity were related to the absence of amino acids supply in cultivation media (Čapková, unpublished data). The evidence of changes in translation apparatus and in RNA distribution induced by the composition of cultivation media was given by Tupý *et al.* (1986). In the RNA-sedimentation spectra of 1 h and 12 h cultivated pollen tubes (Fig. 2) polysomal and monosomal peaks were high and similar, but 12-h-old pollen tubes contained surprisingly more RNPs than those 1-h-old. To confirm this finding, the postmitochondrial supernatant was centrifuged through 60 % sucrose cushion to separate polysomal pellets from postpolysomal supernatant enriched with free RNPs. Absolute amounts of total RNA corresponded to those previously published (Tupý 1982) and relative contents of total RNA in the polysomal fraction and the postpolysomal fraction containing free RNPs were compared. The relative content of total RNA in the postpolysomal fraction increased from 53 % after 1 h of cultivation to 56 % after 4 h, 67 % after 12 h and 69 % after 48 h. These data documented the tendency towards the slow but continuous re-distribution of mRNA from polysomes to postpolysomal fraction enriched with free RNPs during pollen tube growth associated with the decrease of translational efficiency under given cultivation conditions.

Informational content of mRNA populations: RNA isolated from postpolysomal fraction enriched with mRNPs and from polysomes of several developmental stages of tobacco male gametophyte were translated *in vitro* in heterologous system and radioactively labeled *de novo* synthesized polypeptides were then separated by 1-D SDS-PAGE. Differences between protein patterns corresponding to both mRNA fractions in immature

pollen grains were more dramatic than those in pollen tubes (Fig. 3). At all stages of tobacco male gametophyte ontogeny most mRNAs were present in both fractions. However, mRNAs present specifically or preferably in free RNPs or bound to polysomes were identified (Fig. 3).

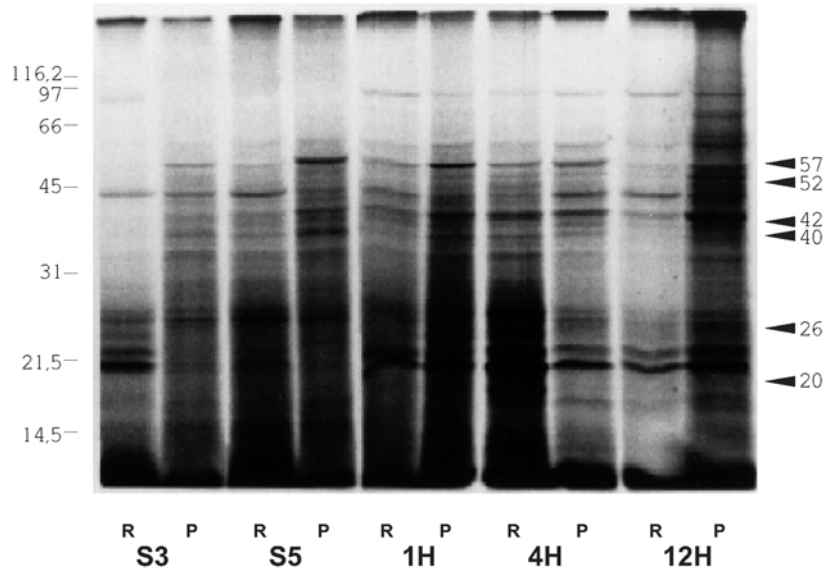

Fig. 3. Differences in polypeptides synthesized *in vitro* in the presence of 10 μg RNA isolated from postpolysomal fraction enriched with mRNPs (R) and polysomal fraction (P) of immature pollen at stages 3 (S3) and 5 (S5) and of pollen tubes cultivated for 1 h (1H), 4 h (4H) and 12 h (12H). Polypeptides synthesized on mRNAs with differential distribution are marked by *arrowheads* with molecular mass [kDa].

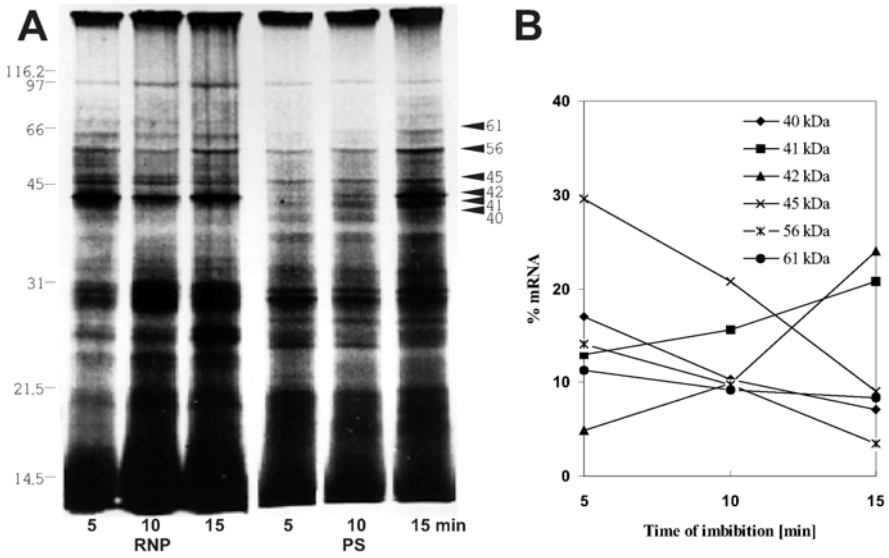

Fig. 4. Dynamics of mRNA association with polysomes during pollen imbibition. Polypeptides synthesized *in vitro* in the presence of 10 μg RNA isolated from postpolysomal fraction enriched with mRNPs (RNP) and polysomal (PS) fractions of pollen imbibed for 5, 10 and 15 min (*A*). Polypeptides corresponding to six particular mRNAs are marked by arrowheads. Their relative abundance at three stages of imbibition was calculated by integration of peaks on densitometrical scans of fluorograms (*B*).

During imbibition, mRNAs were rapidly translocated from stored mRNPs to polysomes (Fig. 2). Remarkable changes were seen in the dynamics of association of different mRNAs with polysomal complexes (Fig. 4). While most mRNAs became associated in the first 5 min of cultivation, others were translocated more slowly within 15 min. No qualitative changes were seen in the population of mRNA from free mRNPs during the first 15 min of pollen cultivation.

The lower translational activity of RNA isolated from postpolysomal fraction and from polysomes compared to samples, where total RNA was used as a template (data not given) may be caused by translational repression in heterologous system as suggested by Minich *et al.* (1989). In order to determine the possible influence of

heterologous translation factors present in rabbit reticulocyte lysate, we compared this *in vitro* translation system with semihomologous system, in which whole polysomal fraction was translated in rabbit reticulocyte system without purification of RNA (Fig. 5). The most interesting finding was significantly higher translational efficiency of semihomologous samples, documented by about 10 times higher incorporation of [^{35}S]-methionine than in samples, where purified RNA was translated (data not shown). There were no dramatic changes in spectra of translated mRNAs, but the relative intensity of particular bands was changed. mRNA encoding for 45 kDa protein became the most efficiently translated in semihomologous system, whereas several others were weakened (37, 54, 60 kDa) or even disappeared (81 kDa at stage 5, Fig. 5). These proteins with varied band intensity could represent mRNAs, expression of which is translationally controlled in germinating pollen, because they probably need factor(s) lacking in rabbit reticulocyte translation system for efficient translation, but this finding is to be subject to further investigation.

In summary, stored mRNA is synthesized during pollen maturation and accumulated in the form of free messenger ribonucleoprotein particles. Pollen activation is closely connected to rapid association of mRNAs with polysomes. Differences in the dynamics of association of particular mRNAs with polysomes suggest that other mechanisms along with rehydration are involved in the pollen activation process. The mRNA storage pool is exploited during pollen tube growth, when translationally repressed mRNA is continuously activated and re-distributed from free RNPs to polysomes. Differences in mRNA populations of free mRNPs and polysomes imply that mRNA re-distribution in pollen tubes is subject to a regulation as an answer to the heterogeneity of the pollen tube pathway through stigma, stylar and placental transmitting tissue. This regulation is possibly involved in changes in the local rate of growth and composition of the pollen tube wall. In contrast to other translationally regulated systems known (ovules, embryos) the role of free mRNPs in the male gametophyte is to maintain translational activity of pollen tubes after the shutdown of transcription.

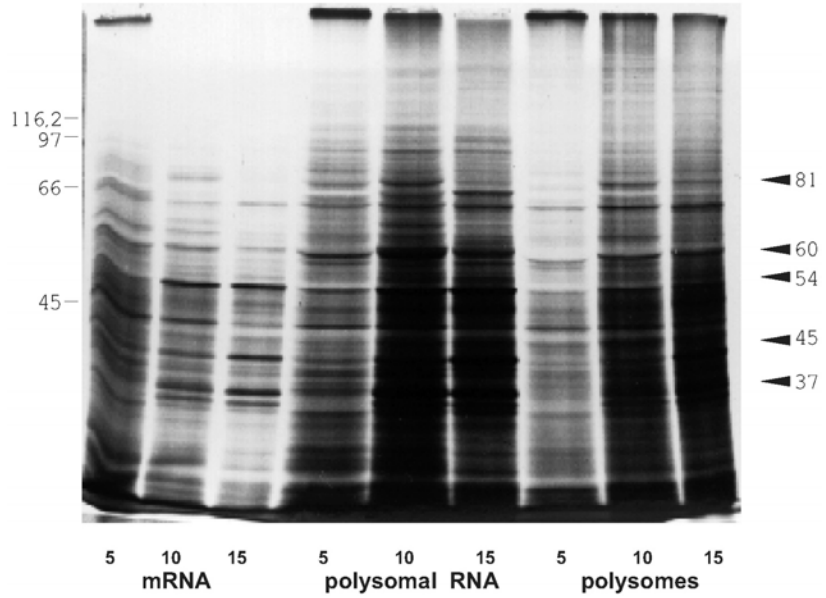

Fig. 5. Semihomologous *in vitro* translation system. Comparison of translation activity *in vitro* in the presence of 10 μg polysomal mRNA, 10 μg RNA isolated from polysomes and of intact polysomes containing 10 μg RNA isolated from pollen imbibed for 5, 10 and 15 min.

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