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The translationally repressed pollen-specific *ntp303* mRNA is stored in non-polysomal mRNPs during pollen maturation

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Abstract A tobacco pollen tube glycoprotein, p69 is encoded by the pollen-specific gene *ntp303* that is transcribed during pollen development and pollen tube growth, but it is abundantly translated only after pollen germination. To investigate the translational repression of *ntp303* mRNA during pollen development the compartmentation of *ntp303* mRNA was examined and compared against another transcript (*ntp52*), which is efficiently translated during pollen maturation. Three subcellular fractions were isolated: a post-polysomal fraction enriched with messenger ribonucleoprotein particles, a polysomal fraction and a novel fraction of EDTA/puromycin-resistant particles co-sedimentating with polysomes (EPP). At all developmental stages studied, *ntp303* mRNA was found to be present in all fractions. Surprisingly, most of the translationally inactive *ntp303* mRNA was localised in the polysomal fraction and EPPs, whereas *ntp52* mRNA was distributed between the post-polysomal fraction and polysomes but was virtually undetectable in EPPs. This differential mRNA distribution pattern may help to explain the developmentally regulated translational repression of the *ntp303* gene during pollen maturation, highlighting a potential role of EPPs. A model of how this differential mRNA compartmentation pattern regulates *ntp303* mRNA translation is proposed.

Keywords Pollen development · Pollen-specific *ntp303* gene · Stored mRNA · Subcellular distribution · Translational regulation · Translational repression

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Introduction

The progamic phase of male gametophyte development is initiated upon pollen hydration and involves activation of synthetic and catabolic processes required for rapid growth of the pollen tube. To achieve this explosive growth the pollen grain accumulates and stores large amounts of both protein and RNA. It is well established that both transcription and translation play an important role in global and specific gene expression patterns during pollen maturation. On the contrary, the germination of many pollen species has been shown to be largely independent of transcription but vitally dependent on translation (see Twell 1994).

In all species studied, levels of total mRNA accumulate progressively during pollen development (Frankis and Mascarenhas 1980; Tupý 1982; Schrauwen et al. 1990). Similarly, the accumulation of mRNA during pollen maturation has been demonstrated for a number of so-called late pollen-specific transcripts (Hanson et al. 1989; Twell et al. 1989; Brown and Crouch 1990; Weterings et al. 1992). The late accumulation of such mRNAs and the presence of full-length transcripts in germinating pollen grains and in pollen tubes demonstrates that many mRNAs survive the pollen dehydration and rehydration processes. Furthermore, the spectra of de novo synthesized proteins in in-vitro-grown pollen tubes have been shown to be similar to those obtained by in vitro translation of mRNA isolated from mature pollen (Mascarenhas et al. 1984; Hussey and Wakeley 1994). Taken together these data provide compelling evidence that many mRNAs are stored in mature pollen grains and translated during germination and tube growth.

Translationally inactive mRNAs have frequently been found associated with a number of proteins forming stored messenger ribonucleoprotein particles (stored mRNPs). Such mRNPs were first described in animal systems (Spirin and Nemer 1965) for which most information is available (Bag 1991; Meric et al. 1996). In plants, stored mRNAs encoding storage proteins in alfalfa embryos were found to be sequestered in the form of

free mRNPs (Pramanik et al. 1992) as well as mRNAs encoding ribosomal proteins in maize embryonic axes (Beltrán-Peña et al. 1995). The presence of specific non-polysomal mRNPs has also been reported in heat-stressed carrot (Apuya and Zimmerman 1992) and tomato cells (Stuger et al. 1999) and in water-stressed *Tortula ruralis* gametophytes (Wood and Oliver 1999).

The translational repression of stored mRNAs in immature pollen has been suggested previously (Mascarenhas 1993). This was directly demonstrated by Štorchová et al. (1994), but evidence for the presence of stored mRNPs in developing pollen is still lacking. There are two well-documented examples of translational regulation in pollen: translational repression of tobacco p69 mRNA (Štorchová et al. 1994) and translational enhancement of tomato *lat52* mRNA (Bate et al. 1996). The tomato anther-specific gene *lat52* encodes an essential allergen-related cysteine-rich protein, which is abundant in mature pollen (Muschietti et al. 1994). *Lat52* mRNA is abundantly expressed during pollen maturation (Twell et al. 1989) and in addition to being regulated at the transcriptional level, the 5'-untranslated region of *lat52* mRNA has been shown to act as a developmentally regulated pollen-specific translational enhancer (Bate et al. 1996).

The tobacco pollen tube wall-specific protein p69 is the major newly synthesised protein in germinating pollen and growing pollen tubes (Čapková et al. 1987, 1988). p69 is a glycoprotein originating by N-glycosylation of a 58-kDa precursor, as documented by coupled in vitro translation/glycosylation experiments (Štorchová et al. 1994) and by in vivo protein synthesis in pollen tubes growing in the presence of tunicamycin, an inhibitor of N-glycosylation (Čapková et al. 1994, 1997). On SDS-PAGE p69 is not present in mature pollen but becomes detectable in pollen tubes 4 h after germination (Čapková et al. 1988). More sensitive in vivo labelling experiments demonstrated p69 synthesis within 1 h of pollen germination (Štorchová et al. 1994). Recently, using the most sensitive in situ immunolocalisation techniques, small amounts of p69 have been detected in developing pollen, documenting its translation at a very low level (Wittink et al. 2000). The observation that inhibition of RNA synthesis in pollen tubes does not influence p69 synthesis (Čapková et al. 1988), together with the discovery of a high level of p69 mRNA in immature pollen by in vitro translation (Štorchová et al. 1994), clearly showed the phenomenon of translationally repressed or stored mRNAs in developing pollen.

Recently, purification and N-terminal amino acid sequencing demonstrated that p69 is encoded by the previously isolated late pollen-specific gene *ntp303* (Weterings et al. 1992; Wittink et al. 2000). To date, p69/*ntp303* represents the only known example of a highly abundant pollen-specific mRNA, which is stored in mature pollen in an untranslated form for subsequent use during pollen tube growth. *Ntp303* therefore represents a good model for study of the mechanisms of developmentally regulated translational repression and the localisation of stored mRNA.

Two central questions are how translation of the abundant *ntp303* mRNA is repressed during pollen maturation and how subsequently *ntp303* mRNA translation is activated during pollen germination. Here we report the development and application of subcellular fractionation methods to investigate the distribution of *ntp303* mRNA in developing pollen. We show that during pollen maturation *ntp303* mRNA and the tobacco *lat52* homologue *ntp52* mRNA (Twell, unpublished) are predominantly associated with the polysomal fraction. However, within the polysomal fraction we have identified a novel subfraction which is resistant to polysome destabilising conditions and contains a significant portion of *ntp303* mRNA, but does not contain *ntp52* mRNA. The distribution of *ntp303* mRNA in the resistant fraction is discussed in relation to a potential model of translational repression.

Materials and methods

Plant material

Flower buds were collected from *Nicotiana tabacum* L. cv. Samsun plants grown under greenhouse conditions. Immature pollen grains at three developmental stages, mid-bicellular (stage 3, bud lengths 26–31 mm), late bicellular (stage 5, bud lengths 48–51 mm) and 1 day before anthesis (stage 6, bud lengths 52–57 mm; Tupý et al. 1983) were isolated from anthers according to Tupý (1982). Mature pollen was collected under aseptic conditions as described previously (Petrů et al. 1964; Tupý et al. 1977).

Subcellular fractionation

Sedimentation of polysomes was performed according to de Vries et al. (1988) with several modifications. Immature pollen isolated from 20 anthers or 100 mg of mature pollen grains was homogenized in a sterile mortar with a pestle under liquid nitrogen with 200 µl of the appropriate extraction buffer. According to the conditions required, five different extraction buffers were used, each in conjunction with the corresponding gradient buffer. Two polysome-stabilising buffers were used, a low-salt buffer (LS) and high-salt buffer (HS). In experiments where polysome dissociation was required, high-salt extraction buffer was used, cycloheximide was replaced by EDTA (HS+E), puromycin (HS+P) or both EDTA and puromycin (HS+EP). The exact composition of all buffers is shown in Table 1. The crude pollen extracts were transferred to sterile centrifuge tubes supplemented with 7 ml of the appropriate extraction buffer. The sample was left on ice for 15 min to weaken cellular structures and centrifuged twice (23,000 *g*, 10 min, 4°C) to obtain a post-mitochondrial supernatant. The post-mitochondrial supernatant was transferred to sterile 10-ml ultracentrifuge tubes (Beckman Instruments) and centrifuged (Beckman 75Ti, 50,000 rpm, 3 h 20 min, 4°C) through a 60% sucrose cushion in gradient buffer to sediment the polysomal fraction. Composition of the gradient buffers corresponded to the extraction buffer used (Table 1). The supernatant containing the post-polysomal fraction was centrifuged again (Beckman 75Ti, 50,000 rpm, 16 h, 4°C) to separate post-polysomal mRNPs by sedimentation.

Isolation of RNA and proteins

Total RNA and proteins were isolated from both plant tissue and pellets containing ribonucleoprotein particles obtained by subcellular fractionation using the single-step method with TRI-Reagent (Sigma) (Chomczynski 1993) strictly following the manufactur-

Table 1 Composition of extraction and gradient buffers used for the separation of polysomal and postpolysomal ribonucleoprotein particles by ultracentrifugation. See Materials and methods

	LS	HS	HS+E	HS+P	HS+EP
Extraction buffers					
Tris-HCl, pH 9.0	200 mM	200 mM	200 mM	200 mM	200 mM
KCl	25 mM	400 mM	500 mM	500 mM	500 mM
Mg-acetate	60 mM	60 mM	1 mM	1 mM	1 mM
DTT	2 mM	2 mM	2 mM	2 mM	2 mM
PMSF	0.5 mM	0.5 mM	0.5 mM	0.5 mM	0.5 mM
PTE	1%	1%	1%	1%	1%
Cycloheximide	1 mM	1 mM	–	–	–
EDTA, pH 8.0	–	–	50 mM	–	50 mM
Puromycin	–	–	–	0.2 mM	0.2 mM
Sucrose	250 mM	250 mM	250 mM	250 mM	250 mM
Gradient buffers					
Tris-HCl, pH 8.5	40 mM	40 mM	40 mM	40 mM	40 mM
KCl	15 mM	100 mM	200 mM	200 mM	200 mM
Mg-acetate	30 mM	30 mM	1 mM	1 mM	1 mM
DTT	2 mM	2 mM	2 mM	2 mM	2 mM
PMSF	0.5 mM	0.5 mM	0.5 mM	0.5 mM	0.5 mM
Cycloheximide	1 mM	1 mM	–	–	–
EDTA, pH 8.0	–	–	50 mM	–	50 mM
Puromycin	–	–	–	0.2 mM	0.2 mM

er's instructions. Total RNA was dissolved in sterile deionized water when used for in vitro translation, or in deionized formamide when used for northern blot analysis. The amount and purity of isolated RNA was determined spectrophotometrically. Proteins were dissolved in sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% BME), boiled for 5 min and loaded onto SDS-PAGE gels.

In vitro translation

Ten micrograms of cytoplasmic RNA isolated from polysomal and post-polysomal fractions was translated under in vitro conditions using the rabbit reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine according to the manufacturer's instructions. When entire polysomal ribonucleoprotein particles were translated, polysomes were pelleted by ultracentrifugation as described and resuspended in 50 µl of sterilized deionized water. The amount of polysomal RNA was estimated spectrophotometrically by measuring absorbance at 260 and 280 nm. Fractions of polysomes corresponding to 10 µg of polysomal RNA were translated under in vitro conditions using the rabbit reticulocyte lysate system (Promega) according to the manufacturer's instructions.

Protein electrophoresis

Proteins of interest were separated by one-dimensional 12.5% SDS-PAGE gels (12.5% T, 2.7% C for the resolving gel and 3% T, 2.7% C for the stacking gel) according to Laemmli (1970). Ten to 30 µg of unlabelled proteins were loaded per lane and proteins were visualised by silver staining (Blum et al. 1987). Separated proteins were electro-blotted onto Hybond-P PVDF membrane (Amersham) at 100 V for 1 h using the semi-dry blotting apparatus (Amersham) and transfer buffers (cathode buffer: 25 mM Tris-Cl, pH 9.5, 40 mM glycine, 10% methanol; anode I buffer: 300 mM Tris-Cl, pH 10.4, 10% methanol; Anode II buffer: 25 mM Tris-Cl, pH 10.4, 10% methanol). Western blots were developed in the primary monoclonal antibody raised against a nuclear-encoded mitochondrial core protein 2 of the cytochrome c reductase complex in a 1:500 dilution. The antibody was kindly given by Dr. H.-P. Braun from University of Hannover, Germany. After in vitro translation, [^{35}S]methionine-labelled proteins corresponding to 100,000 dpm were loaded per lane. Radioactive proteins on the gel were visualised by fluorography (Čapková et al. 1987).

Northern blot hybridisation

RNA samples were denatured and separated on 1.2% agarose gels made in MOPS-electrode buffer (Ausubel et al. 1989). RNA present in both polysomal and post-polysomal fractions was defined as cytoplasmic RNA. The proportional ratio between RNA isolated from both fractions was determined. Ten micrograms of RNA present in the larger fraction was loaded and RNA isolated from the smaller fraction was loaded according to the determined proportional ratio. Following electrophoresis, RNA was transferred onto Hybond-N+ membrane (Amersham) by capillary blotting (Sambrook et al. 1989) and immobilised by UV cross-linking in a Stratalinker (Stratagene).

Nonradioactive digoxigenin-labelled cDNA probes were prepared by PCR amplification in the presence of 0.4 mM DIG-UTP and membranes were hybridised as described in Neuhaus-Url and Neuhaus (1993). The membranes were exposed to Kodak X-Omat S films to detect the signal emitted by CDP-Star chemiluminescent substrate (Tropix).

Radioactively labelled cDNA probes were prepared by random priming (PrimeIT II random primer labelling kit, Stratagene; Feinberg and Vogelstein 1984) in the presence of [α^{32}P]dCTP and purified through NucTrap probe purification columns (Stratagene). Prehybridisation and hybridisation were carried out at 65°C in 20 mM sodium-phosphate buffer, pH 7.4 (Church and Gilbert 1984). Signals were detected using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and quantified using ElfoMan software (Ing. Semecký).

Results

ntp303 mRNA is distributed predominantly in the polysomal fraction

To investigate subcellular distribution of *ntp303* mRNA during tobacco microgametogenesis, two RNA-containing fractions, polysomal and post-polysomal, were separated by centrifugation of the post-mitochondrial supernatant through a 60% sucrose cushion in high salt buffer (HS, for details see Materials and Methods and Table 1). The efficiency of separation of both subcellular fractions

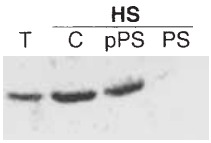

Fig. 1 Western blot immunodetection of 50-kDa nuclear-encoded mitochondrial core protein 2 of the cytochrome c reductase complex within four protein fractions isolated from immature pollen at developmental stage 5. *T* Total proteins; *C* cytoplasmic fraction; *pPS* post-polysomal fraction; *PS* polysomal fraction isolated with high salt buffer (*HS*)

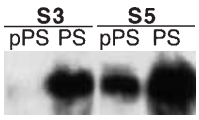

Fig. 3 Northern blot hybridisation of polysomal and post-polysomal mRNAs isolated with HS from immature pollen at stages 3 and 5 with DIG-labelled *ntp303* cDNA probe. The ratio of RNA isolated from both fractions was determined. Ten µg of RNA from the larger fraction was loaded; loading of RNA isolated from the smaller fraction was in the ratio determined for total RNA distribution. Abbreviations as in Fig. 1

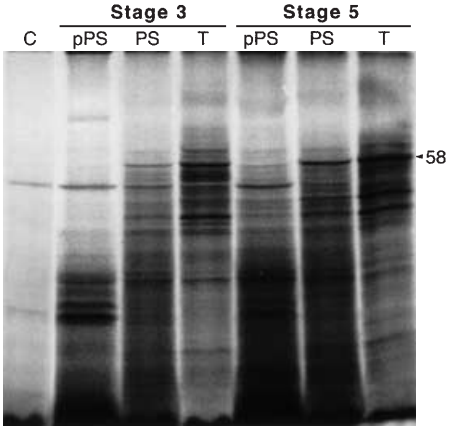

Fig. 2 In vitro translation of polysomal and post-polysomal mRNAs isolated with HS from immature pollen at stages 3 and 5. The *arrowhead* indicates the position of the 58 kDa precursor of p69. *C* – control reaction without added mRNA; *pPS* post-polysomal fraction; *PS* polysomal fraction; *T* total RNA

was tested by immunodetection of 50-kDa nuclear-encoded mitochondrial core protein 2 of the cytochrome c reductase complex within total and cytoplasmic proteins and proteins isolated from both polysomal and post-polysomal fractions from immature pollen at developmental stage 5 (Fig. 1). This protein, synthesised in the cytoplasm, was detected in all cytoplasmic (sub)fractions, including in the post-polysomal, but not in the polysomal fraction. This result documented that polysomes isolated by this method are free of general cytoplasmatic proteins.

In mid- and late bicellular pollen (stages 3 and 5), 28% of cytoplasmic RNA was found in the post-polysomal fraction and 72% in the polysomal pellet. mRNA profiles of separated subcellular fractions were also analysed by in vitro translation. Ten micrograms of cytoplasmic RNA isolated from both polysomal and post-polysomal fractions from developing pollen at stages 3 and 5 was translated in vitro in rabbit reticulocyte lysate and resolved by one-dimensional (1-D) SDS-PAGE (Fig. 2). Differences were detected in translational profiles between mRNA populations present in polysomal and post-polysomal fractions. These differences were more significant in the later stage of pollen development, which may be explained by both the presence of more stored messages at this stage and a more efficient RNA isolation procedure. These data suggested that, under conditions used, the mRNA encoding a 58-kDa protein, the precursor of p69 (Čapková et al. 1994, Štorchová et al. 1994, Čapková et al. 1997), is preferentially present in the polysomal fraction.

This result was further investigated by direct detection of *ntp303* mRNA in northern blot hybridisations of RNA isolated from polysomal and post-polysomal fractions with a DIG-labelled *ntp303* cDNA probe (Fig. 3). RNA was isolated and quantified by spectrophotometry and for two developmental stages it was run on agarose gels. Because of the different content of rRNA, mRNA and tRNA in separate fractions and in order to visualise the relative *ntp303* mRNA distribution between both fractions, directly on the blot, RNA was loaded in the same proportion of polysomal and post-polysomal fractions as determined. The presence of 98% of *ntp303* mRNA in the polysomal fraction at stage 3 and 79% at stage 5 confirmed the data shown in the in vitro translation experiments, that *ntp303* mRNA was distributed preferentially in the polysomal fraction in immature pollen.

Further subcellular fractionation

These data led us to develop more efficient methods for subcellular fractionation to enable the separation of more than two compartments. All results presented in this section were obtained from developing pollen at stage 5. We compared five different extraction buffers, low salt buffer (LS), high-salt buffer (HS) and high-salt buffer supplemented with components known to destabilize polysomal complexes: 50 mM EDTA (HS+E), 0.1 mM puromycin (HS+P), and both 50 mM EDTA and 0.1 mM puromycin (HS+EP) (Infante and Graves 1971; Mansfield and Key 1988; Pastori and Schoenberg 1993). In the presence of polysome-destabilizing agents in HS extraction buffer there were no obvious changes in the mass of the post-polysomal fractions obtained. The polysomal fraction was significantly reduced but never completely disappeared, suggesting the existence of heavy ribonucleoprotein particles resistant to EDTA and/or puromycin treatment (Fig. 4). This finding was confirmed by 1-D SDS-PAGE of proteins purified from polysomal and post-polysomal fractions isolated with all buffers (Fig. 5). Compar-

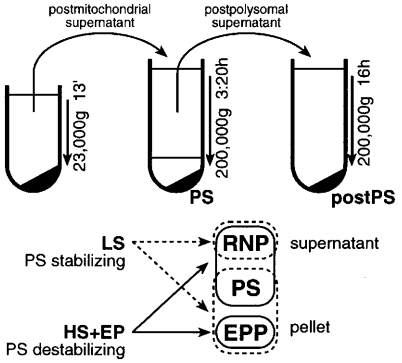

Fig. 4 Subcellular fractionation method. For details see Materials and methods. Under low salt conditions (*LS*) both polysomes and *EPPs* (EDTA/puromycin-resistant particles) sedimented through a sucrose cushion, leaving only post-polysomal *RNP*s (ribonucleoprotein particles) in the supernatant. Treatment with polysome destabilizing compounds (*HS+EP*) led to release of ribosomal subunits to the supernatant

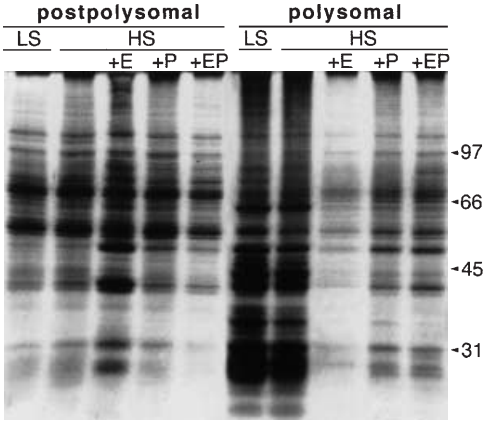

Fig. 5 SDS-PAGE of proteins isolated from post-polysomal and polysomal fractions of immature pollen at stage 5 with five extraction buffers. *LS* low salt buffer; *HS* high salt buffer; *HS+E* high salt buffer supplemented with EDTA; *HS+P* high salt buffer supplemented with puromycin; *HS+EP* high salt buffer supplemented with EDTA and puromycin

ison of the protein spectra demonstrated that both EDTA and puromycin are sufficient to dissociate polysomes. Together with high-salt conditions EDTA and puromycin are likely to disrupt these structures to the extent that they do not co-sediment with post-polysomal ribonucleoprotein complexes, because no significant changes in the protein composition of post-polysomal RNPs were observed. Particles co-sedimenting with polysomes but resistant to EDTA and puromycin treatment, based on their protein composition, seem to form an independent fraction. These particles were therefore named EDTA/puromycin-resistant particles (EPP) and showed characteristic protein profiles visualised by silver staining (Fig. 5). The protein profile of the resistant fraction was more similar to that of post-polysomal particles than to polysomes but was not identical to either of them. The *ntp303* mRNA distribution among all fractions separated was investigated by northern blot hybridisation with a DIG-labelled *ntp303* cDNA probe (Fig. 6). Under both low-salt and high-salt conditions in the presence of cycloheximide (a polysome-stabilising compound), most *ntp303* mRNA was detected in the pelleted polysomal fraction. Treatment of samples with both EDTA and puromycin led to redistribution of part of the *ntp303* mRNA into the post-polysomal fraction. After that treatment only 12% of the cytoplasmic RNA persisted in the EDTA/puromycin-resistant sub-fraction co-sedimenting with polysomes, but this sub-fraction contained more than 50% of the *ntp303* signal. These data show that the fraction of heavy EDTA/puromycin-resistant particles is highly enriched with *ntp303* mRNA.

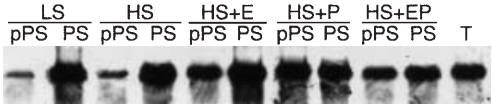

Fig. 6 Northern blot hybridisation of mRNA isolated from post-polysomal and polysomal fractions of immature pollen at stage 5 with five extraction buffers. The ratio of RNA isolated from the two fractions was determined. Ten µg of RNA from the larger fraction was loaded. Loading of RNA isolated from the smaller fraction was at the ratio determined for total RNA distribution. The membrane was hybridized with a DIG-labelled *ntp303* cDNA probe. *Abbreviations* as Figs. 1 and 4

This was further investigated in in vitro translation experiments, where the translatability and informational content of mRNA isolated from the polysomal fraction with LS and HS were compared to those of mRNAs isolated from the EDTA/puromycin-resistant fraction (Fig. 7). Spectra of proteins de novo synthesized were similar in all three samples, but the band corresponding to the polypeptide with apparent molecular weight 58 kDa was significantly stronger in the EDTA/puromycin-resistant fraction. This protein represents the non-glycosylated form of p69 (Čapková et al. 1994, 1997; Štorchová et al. 1994). These data further confirm that *ntp303* mRNA is enriched within the EDTA/puromycin-resistant fraction.

Translatability of the mRNAs was compared with that of the same populations of mRNAs remaining associated with proteins forming mRNPs (Fig. 7). The translatability of mRNPs isolated with LS and HS was comparable to that of the corresponding purified mRNAs, but mRNPs present in the EDTA/puromycin-resistant fraction were not translated at all. To investigate the possibility that residual puromycin from the extraction buffer inhibits translational activity of EPPs, samples containing a mixture of EPPs and polysomal RNPs isolated with HS buffer in various ratios were translated together (Fig. 8). Samples containing at least 10% of polysomal RNPs were successfully translated, excluding the possibility that translational repression of RNA present in EPPs was caused by residual puromycin from the extraction procedure. This observation, together with the protein composition of EDTA/puromycin RNP particles (Fig. 5), strongly suggests that the EDTA/puromycin-resistant fraction may be considered an independent subcellular compartment which does not con-

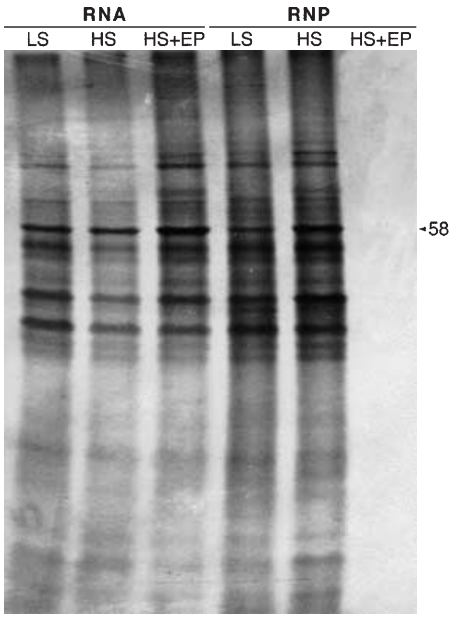

Fig. 7 In vitro translation of polysomal *RNA* and polysomal *RNP*s isolated from immature pollen at stage 5 with three extractions buffers. In *RNA lanes*, purified polysomal RNA was used as a template in the reaction. In *RNP lanes*, whole polysomal RNP particles dissolved in water were translated. The *arrowhead* indicates the position of the 58-kDa precursor of p69. Abbreviations as in Fig. 4

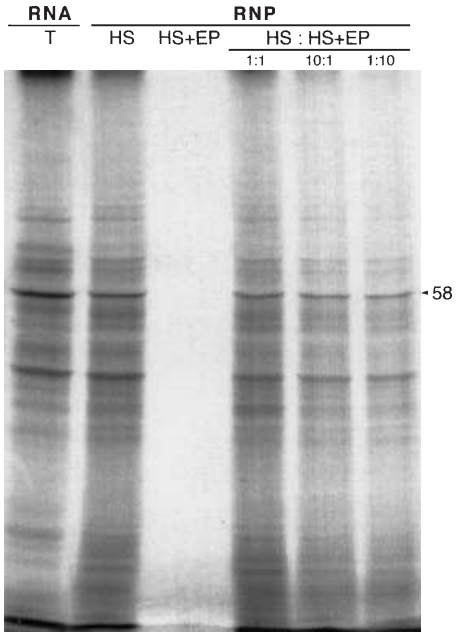

Fig. 8 In vitro translation of polysomal *RNP*s isolated from immature pollen at stage 5 with *HS* and *HS+EP* exctraction buffers. In the *RNA lane* total RNA was used as a template in the reaction. In *RNP lanes* whole polysomal RNP particles dissolved in water were translated. In *HS:HS+EP lanes* polysomal RNP particles isolated with both HS and HS+EP extraction buffers were mixed at determined ratios. The *arrowhead* indicates the position of the 58-kDa precursor of p69. Abbreviations as in Fig. 4.

tain translatable polysomes, but rather contains mRNAs translationally repressed by associated proteins.

Developmental regulation of *ntp303* mRNA subcellular distribution

Based on this experimental data, three cytoplasmic compartments containing translatable mRNA were distinguished in immature pollen grains: polysomal complexes, post-polysomal ribonucleoprotein (RNP) particles and ribonucleoprotein particles resistant to EDTA/puromycin treatment co-sedimenting with polysomes. In the quantitative evaluation, the post-polysomal RNP particles sedimenting in the LS buffer were defined as post-polysomal RNPs. The RNP particles sedimenting in the polysomal fraction in HS buffer supplemented with EDTA and puromycin, were defined as EDTA/puromycin resistant RNP particles, and the RNP particles sedimenting in the polysomal fraction in the LS buffer minus EDTA/puromycin-resistant RNPs were defined as polysomes.

In this developmental study, subcellular distribution of *ntp303* mRNA was compared with mRNA encoding another translationally regulated pollen-specific gene, *ntp52*, by northern blot hybridisation with [^{32}P]-labeled cDNA probes (Fig. 9). At all stages studied the high-salt treatment led to a slight increase of cytoplasmic RNA content in the post-polysomal fraction when compared to low-salt conditions; the increase ranged from 3–19% of total RNA. However, striking changes were observed when high-salt buffer supplemented with EDTA/puromycin (HS+E) was used. Then, a further 31–48% of the RNA was released from the polysomal fraction and the relative RNA content in EDTA/puromycin-sensitive polysomes decreased during development. This corresponds to the previous finding that overall translational activity declines at later stages of pollen development (Tupý et al. 1983). On the other hand, at all developmental stages the ratio of RNA present in EDTA/puromycin resistant RNP particles remained very stable, ranging from 12–16% of total RNA.

At stage 3, most *ntp303* mRNA was present in the polysomal fraction under both low-salt and high-salt condtions (99% and 98%). EDTA/puromycin treatment led to release of 44% of the *ntp303* mRNA from polysomal into the post-polysomal fraction, leaving 54% of the *ntp303* mRNA in EDTA/puromycin-resistant RNP particles. At stages 5 and 6, closer to maturity, the *ntp303* mRNA content in the post-polysomal fraction gradually increased, up to 23% at stage 6, under high-salt conditions, but a relatively high portion (50% and 56%) remained in the EDTA/puromycin-resistant fraction. The *ntp303* mRNA content of polysomal and post-polysomal fractions reached its maximal level in mature pollen, where 86% of this mRNA was present in that relatively small fraction, leaving only 14% in EDTA/puromycin-sensitive structures.

Ntp52 mRNA showed a completely different subcellular distribution during pollen development. It was always found in the post-polysomal fraction at a higher

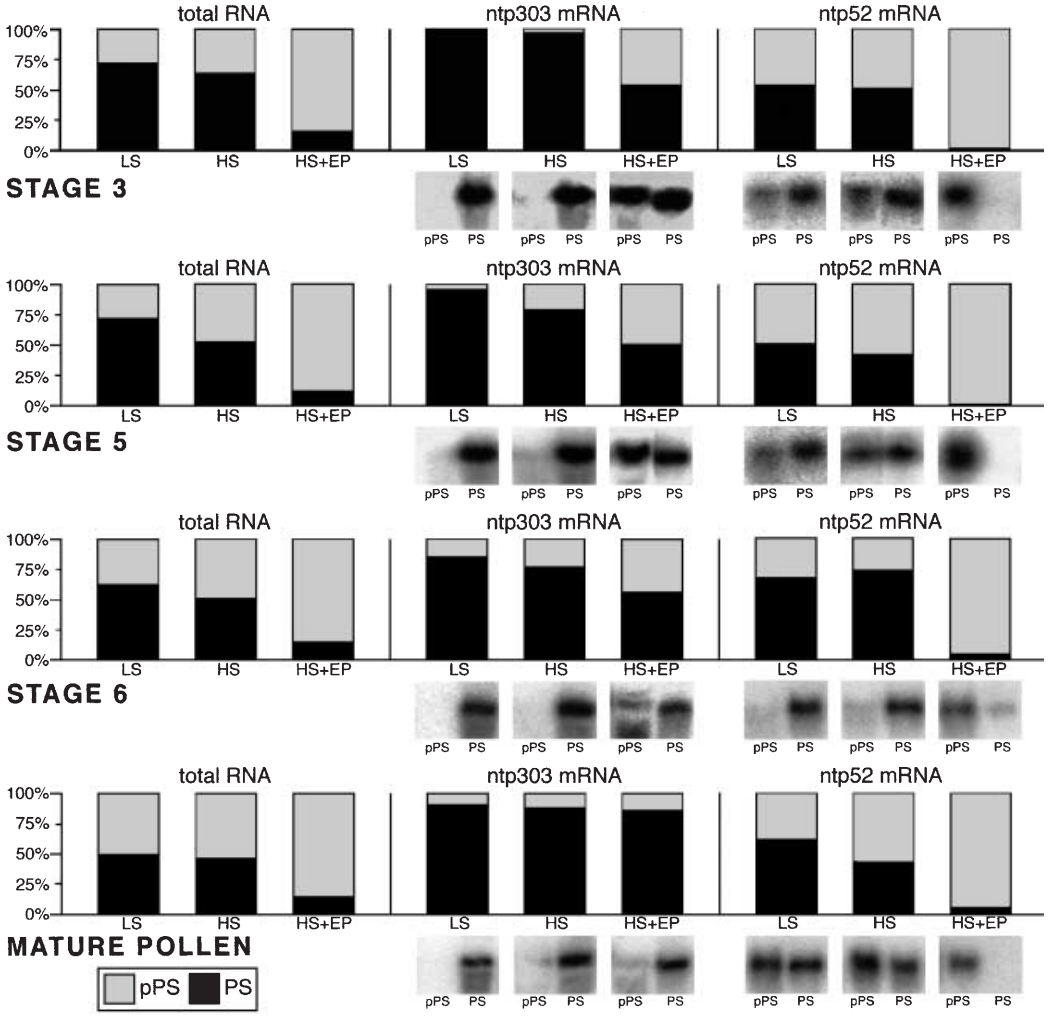

Fig. 9 Developmental regulation of *ntp303* and *ntp52* mRNA subcellular distribution. The *first column* shows total RNA at each stage. Each stage also shows the ratio of RNA isolated from both fractions. For the northern blot analysis 10 µg of RNA from the larger fraction was loaded; loading of RNA isolated from the smaller fraction was in the ratio determined for total RNA distribution. Membranes were hybridized with radioactively labelled *ntp303* and *ntp52* cDNA probes. Quantified signals help visualize the relative mRNA distribution between post-polysomal and polysomal fractions. Abbreviations as in legends of Figs. 1 and 4

level, ranging from 32–49%, with the minimal value at stage 5. EDTA/puromycin treatment very efficiently released most of the *ntp52* mRNA from the polysomal fraction leaving a negligible portion of the mRNA in the EDTA/puromycin-resistant fraction.

The data presented are summarised in Fig. 10, where the relative content of cytoplasmic RNA within all three subcellular fractions separated during pollen development is compared to the distribution of both *ntp303* and *ntp52* mRNA. This figure clearly shows a relatively stable EDTA/puromycin-resistant fraction, an increasing portion of cytoplasmic RNA present in post-polysomal mRNP particles and a corresponding decrease in the ratio of polysomes. *Ntp52* mRNA was found predomiately in ED-

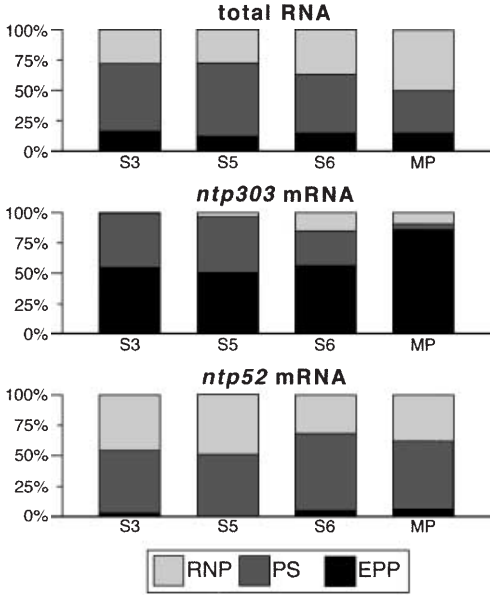

Fig. 10 Summary of *ntp303* and *ntp52* mRNA relative distribution among three subcellular fractions studied during pollen development. *S3* Stage 3; *S5* stage 5; *S6* stage 6; *MP* mature pollen. *RNP* Post-polysomal RNPs; *PS* polysomes; *EPP* EDTA/puromycin resistant particles co-sedimentating with polysomes

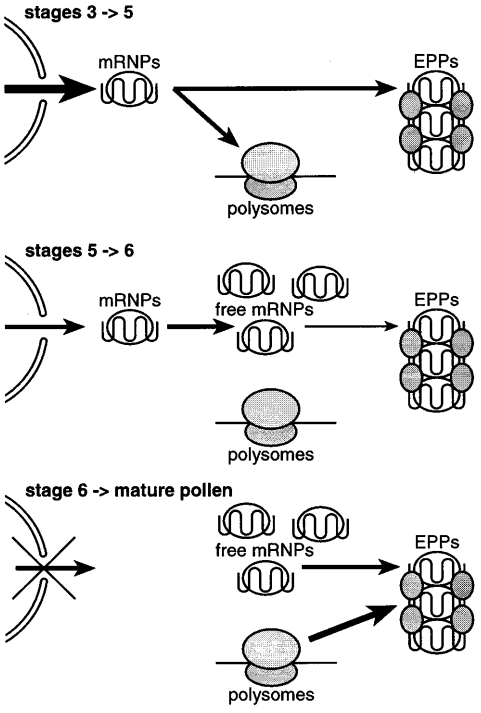

Fig. 11 Potential model of the developmental regulation of *ntp303* mRNA subcellular distribution. Newly synthesized *ntp303* mRNA is released from the nucleus in the transport form of *mRNPs*. *Between stages 3 and 5 ntp303* mRNA is distributed evenly between polysomes and EPPs. All polysomes associated with *ntp303* mRNA are formed at this stage and are translationally silent. *EPPs* are proposed to be the long-term storage compartment formed by aggregation of individual *ntp303* mRNPs, probably with other proteins. *Between stages 5 and 6*, polysomes associated with *ntp303* mRNA are still present in the vegetative cell but their amount does not increase. *ntp303* mRNA synthesized at this time remains in the transient form of "free" *mRNPs* with only a small portion combining into EPPs. In the final period of maturation, *between stages 6 and mature pollen*, the synthesis of *ntp303* mRNA is complete, but a massive redistribution of *ntp303* mRNA from "free" mRNPs and polysomes to EPPs occurs

TA/puromycin-sensitive RNP particles divided between the polysomal and post-polysomal fractions. In contrast, *ntp303* mRNA, originally present in both the polysomal fraction and the EDTA/puromycin resistant fractions, was redistributed into EDTA/puromycin-resistant RNP particles during the final stages of pollen maturation.

Discussion

Inspired by previous studies documenting the presence of stored mRNA in plant cells, namely in embryos (Pramanik et al. 1992; Beltrán-Peña et al. 1995; Rincón-Guzmán et al. 1998), we looked for post-polysomal messenger ribonucleoprotein particle s (mRNPs) in developing tobacco pollen. Subcellular fractionation methods were used to separate cytoplasmic post-polysomal RNPs from polysomal complexes according to their sedimentation. At stages 3 and 5, a significant portion of RNA was found in the post-polysomal fraction. The protein composition of post-polysomal RNP particles was found to differ from that of polysomal RNPs (Fig. 5), and the polysomal fraction was documented to be free of general cytoplasmic proteins (Fig. 1). Both fractions were found to contain translatable mRNA (Fig. 2), a different set in each compartment. In the in vitro translation system, only polysomal RNPs were translatable (Fig. 7), whereas post-polysomal RNPs were not. mRNA associated with them was likely to be translationally repressed by associated proteins. This post-polysomal fraction is presumed to represent an mRNA storage compartment.

Experiments were focused on further separation of the polysomal fraction. Treatment with the polysome-destabilizing compounds EDTA and puromycin led to identification of a third compartment containing mRNA, EDTA/puromycin-resistant RNP particles co-sedimenting with polysomes (EPP). EPPs form an independent compartment as shown by their protein composition (Fig. 5) and contain translatable mRNA (Fig. 7). Ribonucleoprotein particles present in the polysomal fraction isolated with low-salt and high-salt buffer were translatable in the in vitro translation system (Figs. 7, 8), whereas EPPs were not. mRNA together with associated proteins forming EPPs (Figs. 7, 8) was translationally silent. Based on the similarity in protein composition of EPPs and post-polysomal RNPs, and on translational repression of associated mRNA, EPPs might represent higher order aggregates of post-polysomal RNPs in developing pollen.

Surprisingly, under high-salt conditions, translationally repressed *ntp303* mRNA (Weterings et al. 1992; Storchová et al. 1994) was detected almost exclusively in the polysomal fraction at stages 3 and 5 (Fig. 3). The presence of translationally silent polysomes has been previously reported in stressed potato tubers (Crosby and Vayda 1991). We argue that during the final stages of development pollen may be considered to be under stress conditions. This is documented by previously published high levels of free proline (Stanley and Linskens 1974; Schwacke et al. 1999) and an increase in osmotic pressure by starch hydrolysis in pollen grains close to maturity (Tupý et al. 1992). Under these conditions it would be advantageous for germinating pollen, needing massive protein synthesis immediately after imbibition (see Twell 1994), to have the most important mRNAs associated with polysomes poised for translation. Part of the *ntp303* mRNA originally present in the polysomal fraction was released by increased salt concentration in the presence of cycloheximide. Both EDTA and puromycin each acting alone were more efficient in disrupting polysomal complexes, but the most dramatic effect was observed when both were used together. Under such treatment it was shown by northern blot hybridization (Fig. 6) and in vitro translation (Fig. 7) that the EPP fraction is highly enriched with *ntp303* mRNA. This suggests a more complicated mechanism of *ntp303* translational regulation involving all three characterized subcellular compartments during pollen development.

Distribution of *ntp303* mRNA between the three characterized compartments during pollen development was

further investigated by northern blot hybridization and compared to the distribution in another pollen-specific transcript, *ntp52*. From mid-bicellular stage (stage 3) to pollen maturity total RNA was shown to increase 250%, from 90 pg/cell to 230 pg/cell (Tupý 1982). The highest increase was observed between stages 3 and 5 when pollen grains contain approximately 210 pg RNA/cell. At these developmental stages most RNA (56–60%) was found in the polysomal fraction (Fig. 10). These data correspond with previously documented maximal translational activity between these stages (Tupý et al. 1983). Closer to maturity, as translational activity declines, RNA is gradually redistributed to the post-polysomal fraction. The relative content of this fraction nearly doubles between stage 3 and mature pollen. The EDTA/puromycin-resistant fraction was already formed at stage 3 and its relative RNA content was found to be very stable during pollen development, at 15% of total RNA.

The two pollen-specific transcripts, *ntp303* and *ntp52*, were shown to have completely different distributions during pollen development (Fig. 10). At stage 3, *ntp303* mRNA was evenly distributed between EDTA/puromycin-sensitive polysomes and EPPs. Between stages 3 and 5 all polysomes associated with *ntp303* mRNA were formed; later, this message was present in post-polysomal RNPs and EPPs. During the final stages of maturation, most *ntp303* mRNA was redistributed from the other two compartments to EPPs. The *lat52* gene appears to show a more typical pattern of expression; *lat52* mRNA accumulates during pollen development (Twell et al. 1989) and it is both stored and efficiently translated. Its 5'-UTR contains a strong translational enhancer, dramatically increasing the translational yield, especially during the final stages of maturation (Bate et al. 1996). *Ntp52* transcript was found only in negligible amounts in EPPs; its distribution between polysomes and post-polysomal particles followed the translational profile. The amount of *ntp52* mRNA bound to polysomes reached the maximal level at stage 6 (Fig. 10). In mature pollen, *ntp52* transcript was also found in EDTA/puromycin-sensitive polysomes.

A possible model of *ntp303* mRNA distribution and redistribution during pollen maturation is described in Fig. 11. This model introduces a newly identified subcellular compartment of EDTA/puromycin-resistant particles as possible aggregates of individual post-polysomal RNPs. Although these structures are formed continuously during pollen maturation, *ntp303* mRNA appears to be redistributed from polysomes and post-polysomal mRNPs to EPPs just prior to maturity. We propose that EPPs are formed in order to assist stored mRNA survive dessication. It is notable that *ntp52* mRNA does not appear in these structures. The interesting finding of polysomes with associated mRNA in dehydrated pollen suggests alternative mechanisms of mRNA storage in pollen. Two alternative storage compartments in dry pollen for mRNAs, which are actively translated before germination, such as *ntp52* mRNA, are EDTA/puromycin-sensitive polysomes and post-polysomal mRNPs. Particular mRNAs may be stored in association with polysomal complexes, ready for immediate onset of translation after germination. With regard to *ntp303*, it is now important to discover the *cis*- and/or *trans*-acting factors influencing recruitment of mRNA to translationally silent polysomes and its further redistribution to EPPs.

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