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Cytoskeleton-associated large RNP complexes in tobacco male gametophyte (EPPs) are associated with ribosomes and are involved in protein synthesis, processing, and localization

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Annotation: In the presented work the composition and function of stored ribonucleo-protein particles (EPPs) in tobacco male gametophyte was studied. EPP particles serve as a long-term store of various mRNA molecules, ribosomal and cytoskeletal proteins and other components. They are synthesized during the pollen maturation, kept untranlated and gradually used in proteosynthesis in progamic phase to support the rapid pollen tube growth.

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The coauthors listed below fully acknowledgement that David Reňák performed the major part in the presented work with the mention of his contribution.

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Cytoskeleton-Associated Large RNP Complexes in Tobacco Male Gametophyte (EPPs) Are Associated with Ribosomes and Are Involved in Protein Synthesis, Processing, and Localization

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The progamic phase of male gametophyte development involves activation of synthetic and catabolic processes required for the rapid growth of the pollen tube. 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, germination of many pollen species has been shown to be largely independent of transcription but vitally dependent on translation of stored mRNAs. Here, we report the first structural and proteomic data about large ribonucleoprotein particles (EPPs) in tobacco male gametophyte. These complexes are formed in immature pollen where they contain translationally silent mRNAs. Although massively activated at the early progamic phase, they also serve as a long-term storage of mRNA transported along with the translational machinery to the tip region. Moreover, EPPs were shown to contain ribosomal subunits, rRNAs and a set of mRNAs. Presented results extend our view of EPP complexes from mere RNA storage and transport compartment in particular stages of pollen development to the complex and well-organized machinery devoted to mRNA storage, transport and subsequent controlled activation resulting in protein synthesis, processing and precise localization. Such an organization is extremely useful in fast tip-growing pollen tube. There, massive and orchestrated protein synthesis, processing, and transport must take place in accurately localized regions. Moreover, presented complex role of EPPs in tobacco cytoplasmic mRNA and protein metabolism makes them likely to be active in another plant species too. Expression of vast majority of the closest orthologues of EPP proteins also in *Arabidopsis* male gametophyte further extends this concept from tobacco to *Arabidopsis*, the model species with advanced tricellular pollen.

Keywords: *Nicotiana tabacum* • male gametophyte • pollen • model plant • translation regulation • mRNA storage • cytoskeleton • mRNP • ribonucleoprotein particle

1. Introduction

The functional (progamic) phase of male gametophyte development is initiated upon the pollen hydration on a stigma and involves the activation of processes required for the rapid growth of the pollen tube. To achieve this explosive growth, the pollen grain accumulates and stores large amounts of both RNA and protein reserves. It is a well-documented fact that both transcription and translation play an important role in global and specific gene expression patterns during the pollen maturation. On the contrary, germination of many pollen species has been shown to be largely independent of transcription but vitally dependent on translation.[1,2]

Translationally inactive mRNAs have frequently been found in association with a number of proteins forming stored messenger ribonucleoprotein particles (stored mRNPs). The accumulation of newly synthesized mRNAs in the form of mRNPs was first described in animal cells and such form of translational regulation of gene expression was later documented in a broad spectrum of cell types, from fish and urchin embryos,[3] spermatic cells,[4] oocytes to neuronal cells.[5−9] Translational regulation in plants has been studied to less extent than in animal systems. Stored mRNP complexes were identified in

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wheat embryos.[10] In alfalfa, differences in mRNP accumulation were described in zygotic and somatic embryos,[11] whereas stage-specific mRNPs during embryogenesis were identified by Pramanik et al.[12] Segregation of seed-storage-protein mRNA was described in rice endosperm cells.[13,14] The presence of specific nonpolysomal mRNPs has also been reported in heat stressed carrot[15] and tomato cells[16] and in water-stressed *Tortula ruralis* gametophytes.[17] Moreover, the occurrence of polysomes in cytoskeleton and membrane-bound fractions was intensively studied and the association of ribosomes with cytoskeleton was repeatedly proven.[18−21]

Stored mRNPs were found to be a component of successfully exploited mechanism driving the ontogenetic differentiation of polarized and/or transcriptionally quiescent cells. Moreover, mRNPs are organized into higher-order complexes.[22] Such complexes often contain various components of translational apparatus, both mRNAs and proteins.[5,23] Translational control of individual mRNAs facilitates precise regulation of their trafficking, localization, and storage. Their activation finally requires also exact spatial and temporal expression patterns of regulatory proteins. Mechanisms regulating the complex process of mRNA translational repression and activation within such structures are far from being understood, but RNA-binding proteins and cytoskeleton play a major role in this process. The heterogeneous group of proteins binding to *cis*-elements within RNA has been recently classified into several families.[6] Microtubules are often mentioned as a cytoplasmic railway for translocation of many RNPs, while actin seems to be connected with anchoring and translational activation of RNPs. However, its role in RNP translocation has been described as well.[23,24] Cytoskeleton-dependent localization signals were detected in 3-UTR of several mRNAs.[25,26] The identification of proteins associated with both RNP proteins and cytoskeletal structures suggests the association of RNPs and cytoskeleton in a sequence-independent but protein size- and conformation-dependent manner.[23,25,27,28]

Tobacco male gametophyte represents a well-characterized model of translational regulation. Pollen specific gene *NTP303* encodes p69,[29,30] a major pollen tube wall-specific glycoprotein, which is massively synthesized in germinating pollen and growing pollen tubes.[1,31] On the contrary, *ntp303* mRNA is intensively synthesized during the pollen maturation[32] and is stored in the form of EPP complexes.[33] These mRNP complexes containing translationally silent *ntp303* mRNAs are resistant to polysomal destabilizing components and co-sediment with polysomes.[33] Here, we analyzed the size and structure of large ribonucleoprotein particles (EPP) complexes by means of the size exclusion chromatography and electron microscopy. The association of EPPs with cytoskeleton was determined by fluorescence microscopy and Western blot immunodetection. We also present evidence that EPPs comprise whole ribosomal subunits, a number of translationally silent mRNAs supplemented with a set of various proteins. It documents EPPs' complex role in RNA metabolism and precisely regulated protein synthesis.

2. Material and Methods

2.1. Plant Material. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun) were grown in a greenhouse. Young pollen grains were collected at the middle stage of pollen maturation, when the vegetative cell is filled with cytoplasm and the starch deposition begins. Pollen free of microbial contamination was obtained by surface sterilization of buds on the day of anthesis.[34] The pollen was cultivated under sterile conditions as a shaken suspension at 26 °C and pollen density of 0.5 mg/mL in sugar-mineral medium supplemented with MES-KOH buffer pH 5.9 (SMM-MES)[35] and casein hydrolysate 1 mg/mL. Cultivation intervals were 30 min, 4 or 24 h.

2.2. Isolation of EPP Complexes. EDTA/puromycin-resistant particles were isolated as described previously.[33] Briefly, immature pollen from 20 anthers or 100 mg of mature pollen grains or pollen tubes was homogenized in sterile HSEP buffer (200 mM Tris-HCl, pH 9.0, 500 mM mM KCl, 1 mM Mg-acetate, 2 mM DTT, 0.5 mM PMSF, 1% PTE, 50 mM EDTA, pH 8.0, 0.2 mM puromycin, and 250 mM sucrose) and postmitochondrial supernatant was centrifuged through the 30%, 40%, 50% or 60% sucrose cushion (Beckman L-70, NVT 90, 50 000 rpm, 3 h and 20 min, 4 °C) in HSEP gradient buffer (40 mM Tris-HCl, pH 8.5, 200 mM KCl, 1 mM Mg-acetate, 2 mM DTT, 0.5 mM PMSF, 50 mM EDTA, pH 8.0, and 0.2 mM puromycin). Pelleted EPP complexes or polysomes were washed with deionized water and subjected to further analyses. Alternatively, we employed size exclusion chromatography described below in order to isolate EPP complexes directly from postmitochondrial supernatant.

2.3. Size Exclusion Chromatography. FPLC Äkta Explorer-900 (GE Healthcare Bio-Sciences, Uppsala, Sweden) with computer controlled via UNICORN ver. 4.11. software was used with following characteristics: column hardware XK 16/70, internal diameter 16 mm, length 70 cm, maximal operating pressure 4 bar (72 psi, 0.5 MPa), maximal volume 120 mL, maximal sample load volume 5−10 mL. Superdex 200 prep grade (GE Healthcare Bio-Sciences, Uppsala, Sweden) separation range 10−600 kDa and Sephacryl S-400 HR (GE Healthcare Bio-Sciences, Uppsala, Sweden) separation range 20−8000 kDa were equilibrated with sample buffer (40 mM Tris-HCl, pH 8.5, 200 mM KCl, 1 mM Mg-acetate, 2 mM DTT, 0.5 mM PMSF, and 50 mM EDTA, pH 8.0). Running conditions: temperature 10 °C, injection 1 or 10 mL, flow rate 0.200 mL/min, UV detection 280 nm. The chromatographic performance of SEC columns was determined by injecting a mixture of six proteins used as molecular weight markers in the range 29−669 kDa (Sigma-Aldrich, St. Louis, MO). Good linearity of the calibration curve ($r^2 = 0.991$) over the tested range was obtained. The calibration curve was used for the calculation of the molecular weight of sample components.

Two sample types were applied to the column: (A) fractions of EPP complexes pelleted through the 60%, 50%, 40% or 30% sucrose cushion and resuspended in HSEP homogenization buffer or (B) postmitochondrial supernatant including complete EPP fraction. Five milliliters or 10 mL fractions were collected, dialyzed, lyophilized or desalted, concentrated using Vivapore Q 10 (Vivascience, Hannover, Germany)m and precipitated with 3 vol of ethanol.

2.4. RNA and Protein Isolation. Total proteins and RNA were extracted from EPP complexes and polysomes with TRI-Reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Isolated RNA was dissolved in sterile deionized water and its yield and concentration were determined spectrophotometrically. Proteins were dissolved in SDS-PAGE buffer[36] or 2-D sample buffer (8 M urea, 2% urea, 50 mM DTT, 2% 100× Bio-Lyte; BioRad, Hercules, CA). The yield was determined according to Schaffner et al.[37]

2.5. Protein Electrophoresis. Proteins in EPP complexes were separated by 2-D PAGE on a Protean IEF Cell (Bio-Rad, Hercules, CA; strip size 11 cm, ampholyte pH range 3−10). The second dimension was performed on a Biometra (Whatman-

Biometra, Göttingen, Germany) apparatus using 12% resolving gel. Protein patterns were visualized by silver staining according to Sammons et al.[38] For immunodection, EPP proteins were separated using 1-D SDS-PAGE and transferred to nitrocellulose membrane (NC 45, Serva, Heidelberg, Germany) by blotting. Cytoskeletal proteins were detected using commercially available monoclonal antibodies developed against actin (clone C4, MP Biomedicals, Irvine, CA) and tubulin (TU 01, ExBio, Prague, Czech Republic).

2.6. RT-PCR. Total RNA was extracted from EPP and polysomal pellets using the RNeasy plant kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The yield and RNA purity were determined spectrophotometrically. Samples of 1 μg of total RNA were reverse transcribed in a 20-μL reaction using the ImProm-II Reverse Transcription System (Promega, Madison, WI) following the manufacturer's instructions except that the oligo$(dT)_{15}$ primer was accompanied with custom-synthesized 18S-R and 28S-R primers in order to reverse transcribe 18S and 28S rRNAs together with mRNAs. For PCR amplification, 1 μL of 50× diluted RT mix was used. The PCR reaction was carried out in 25 μL with 0.5 units of Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania), 1.2 mM $MgCl_2$, and 20 pmol of each primer. The PCR program was as follows: 2 min at 95 °C, 33 cycles of 15 s at 94 °C, 15 s at the optimal annealing temperature (55−58 °C), and 30 s at 72 °C, followed by 10 min at 72 °C. As a negative control, complete reverse transcription reaction without reverse transcriptase was used. All primers (Supplementary Table 1 in Supporting Information) were designed using Primer3 software (http://frodo.wi.mit.edu/).

2.7. Electron Microscopy. EPP suspension was dropped on grids with Formvar support layer coated with carbon. Excessive liquid was removed using filter paper. The grid was then negative stained with 2% uranyl acetate. Electron microscopy was performed with JEOL TEM 1011 electron microscope using 80 kV.

2.8. Fluoroscence Analyses of EPPs and Polysomal Complexes. Polysomal and EPP pellets were resuspended in PIPES buffer (50 mM PIPES, 2 mM EGTA, and 2 mM $MgSO_4$) by vortexing for 6 h at 4 °C. The suspension was tested separately for the presence of actin and tubulin using rhodamine-phalloidin and FITC-labeled IgG, respectively. Actin and tubulin were visualized using epifluorescence illumination under a Nikon Eclipse TE2000-E microscope and captured images were processed using Lucia G 4.70 software (Laboratory Imaging, Prague, Czech Republic, www.lim.cz). For actin detection, 100 μL of protein suspension was mixed with 5 μL of rhodamine-phalloidin (Sigma-Aldrich, St. Louis, MO) and the fluorescence was observed after 10 min incubation. For tubulin detection, 100 μL of protein suspension was gently centrifuged at 2500*g* for 90 s, the supernatant was discarded, and the pellet was preblocked with 100 μL of 2% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS: 138 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, and K_2HPO_4, pH 7.4, Sigma-Aldrich, St. Louis, MO) for 30 min. Protein pellet was further incubated with the primary antibody (anti-α-tubulin, clone B-5-1-2, Sigma-Aldrich, St. Louis, MO) at the final dilution of 1:1000 for 2 h and washed three times with PBS. A 2-h treatment with the secondary antibody (anti-mouse IgG-FITC, Sigma-Aldrich, St. Louis, MO) at final dilution of 1:128 was followed by the last wash with PBS. After each step, the suspension was centrifuged at 2500*g* for 90 s in a microcentrifuge tube and the supernatant was replaced with the appropriate solution.

2.9. Mass Spectrometric Analyses. SDS-PAGE gel was cut to 40 slices which were covered with 100 μL of 50 mM ammonium bicarbonate (ABC) buffer in 50% acetonitrile (ACN) with 50 mM dithiotheritol (DTT) and subjected to sonication in an ultrasonic cleaning bath for 5 min. After 15 min, the supernatant was discarded and gel was covered with 100 μL of 50 mM ABC/50% ACN with 50 mM iodoacetamide and sonicated for 5 min and then incubated another 25 min. Supernatant was discarded and exchanged with 100 μL of 50 mM ABC/50% ACN with 50 mM DTT and sonicated 5 min to remove any excess iodoacetamide. Supernatant was discarded and samples were sonicated for 5 min in 100 μL of HPLC water. Water was discarded and samples were sonicated for another 5 min in 100 μL of ACN. ACN was discarded and samples were let open for several minutes to evaporate the rest of ACN. Five nanograms of trypsin (Promega) in 10 μL of 50 mM ABC was added to the gel. Samples were incubated at 37 °C overnight. TFA and ACN were added to reach a final concentration of 1% TFA and 30% ACN to wash out peptides. Eluate was diluted with water (1:2) and subjected to LC-MALDI analyses performed using Ultimate 3000 HPLC system (Dionex) coupled with Probot microfraction collector (Dionex). Tryptic peptides were loaded onto a PepMap 100 C18 RP column (3 μm particle size, 15 cm long, 75 μm internal diameter; Dionex) and separated with a gradient of 5% (v/v) ACN, 0.1% (v/v) trifluoroacetic acid to 80% (v/v) ACN, 0.1% (v/v) trifluoroacetic acid over a period of 45 min. The eluate was mixed 1:3 with matrix solution (20 mg/mL α-cyano-4-hydroxycinnamic acid in 80% ACN) prior to spotting onto a MALDI target. Spectra were acquired on 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems/MDS Sciex) equipped with a Nd:YAG laser (355 nm, firing rate 200 Hz). All spots were first measured in MS mode and then up to 10 strongest precursors were selected for MS/MS which was performed with 1 kV collision energy and operating pressure of collision cell set to 10^{-6} Torr.

Peak lists from the MS/MS spectra were generated by GPS Explorer v. 3.6 (Applied Biosystems/MDS Sciex) and searched by local Mascot v. 2.1 (Matrix Science) against nonredundant protein database NCBI (www.ncbi.nlm.nih.gov, as of June 10th 2008). Database search criteria were as follows: enzyme, trypsin; taxonomy, tobacco (actually searched 2049 sequences); fixed modification, carbamidomethylation; variable modification, methionine oxidation; peptide mass tolerance, 120 ppm; allowed one missed cleavage; MS/MS tolerance, 0.2 Da; maximum peptide rank, 1; minimum ion score C.I. (peptide), 95% ($E \leq 0.05$ for individual MS/MS).

3. Results

3.1. EPPs Are Present in Growing Pollen Tubes. The presence of EPPs in immature and mature pollen was demonstrated previously.[33] During the progamic phase, EPPs were detectable in activated pollen grains after 30 min imbibition in the cultivation medium as well as in growing pollen tubes cultivated *in vitro* for 4 h and even 24 h (Figure 1). 2-D SDS-PAGE gels were loaded with proteins isolated from an equal amount of mature dry pollen (40 mg). This documents gradual but significant 3-fold decrease in EPP abundance during the pollen tube growth. It dropped from 2.4 μg of EPP proteins/1 mg pollen in mature pollen (0.24% fresh weight of mature pollen) to 0.8 μg/1 mg pollen in 24 h-old tubes (0.08%). Moreover, this quantitative decline in EPP abundance was not

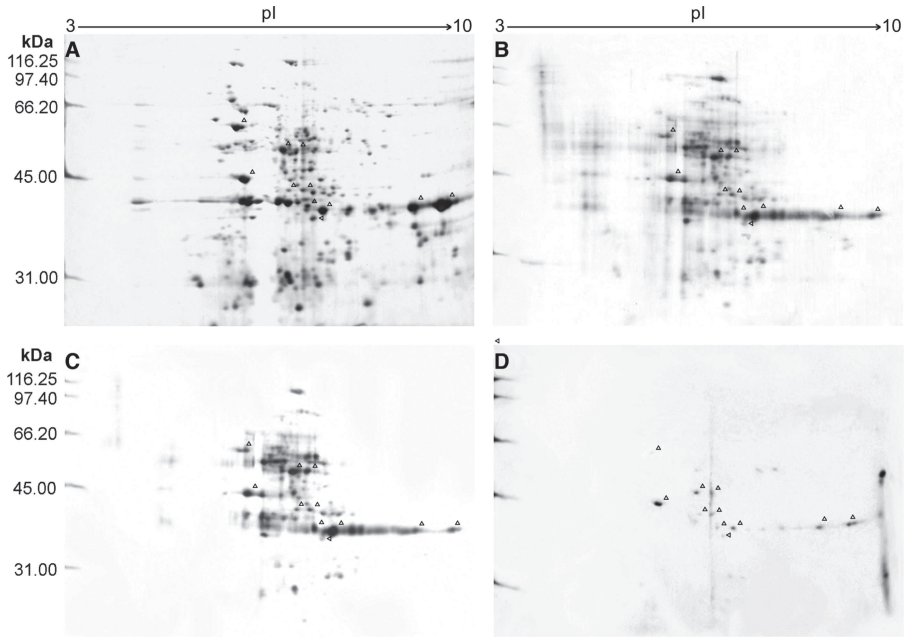

Figure 1. 2D-SDS-PAGE of EPP fraction. EPPs were isolated by HSEP buffer from mature pollen (A) and from pollen tubes cultivated for 30 min (B), 4 h (C) and 24 h (D) in SMM-MES medium. Important proteins present in EPPs throughout the whole cultivation period are marked with arrowheads.

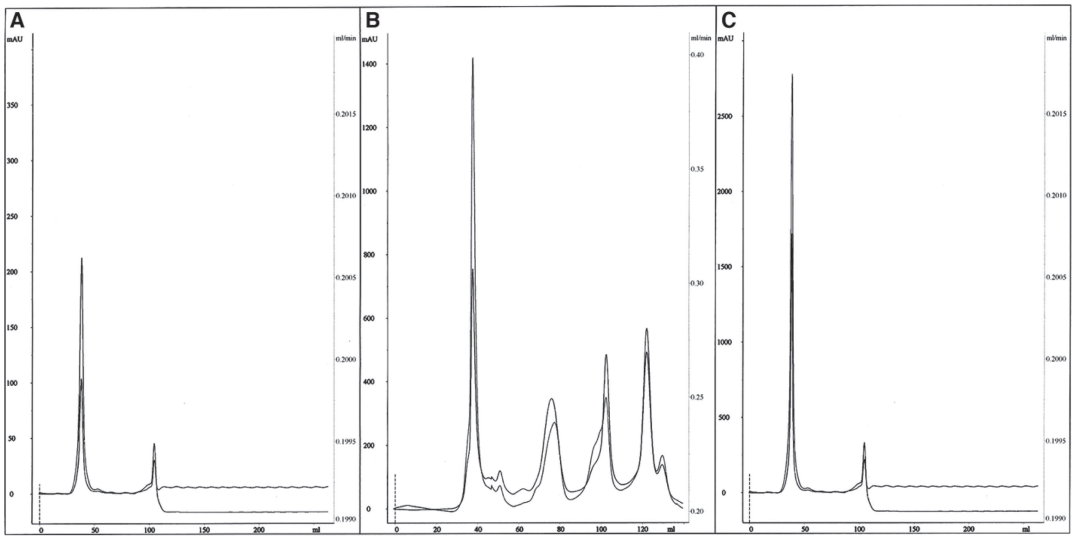

Figure 2. SEC chromatograms of the EPP fractions isolated from mature pollen grains. Samples applied onto the chromatography column: EPP fraction pelleted though 60% sucrose cushion (A), the whole postmitochondrial supernatant (B) and EPP fraction pelleted though 30% sucrose cushion (C).

accompanied by any visible changes in their protein composition, although after 24 h of cultivation only the major protein spots were detectable. However, the qualitative stability of EPP complexes during the whole progamic phase was further documented by comparison of 2-D SDS-PAGE patterns, when an equal amount of 150 μg of proteins was applied per sample (data not shown).

3.2. Size of EPP Complexes. EPP complexes were further analyzed by Size Exlusion Chromatography (SEC). Pollen grains were homogenized in HSEP buffer, which causes efficient dissociation of polysomes by high salt concentration in combination with EDTA and puromycin. Under these conditions, ultracentrifugation of postmitochondrial supernatant through 60% sucrose cushion led to the sedimentation of pure EPP complexes.[33] To determine the apparent molecular weight of EPPs, Superdex 200 prep grade gel with range 10−600 kDa was initially used. Fractions were collected across the separation range of SEC column and the presence of EPPs was investigated by UV absorbance at 280 nm. EPPs were not retained at all, suggesting that they were of molecular weight over 600 kDa. Taking this into account, a second gel type Sephacryl S-400 HR with wider separation range (20−8000 kDa) was packed into the SEC column. EPPs, however, were eluted in the void volume of this gel too. This indicated that EPPs have molecular weight equal to, or higher than, 8 MDa (Figure 2).

With regard to a possible quantitative correlation between the yield of EPPs and ultracentrifugation conditions, SEC was used to evaluate different isolation protocols. The parameters compared included the ultracentrifugation speed and duration as well as various sucrose concentrations in the cushion. In

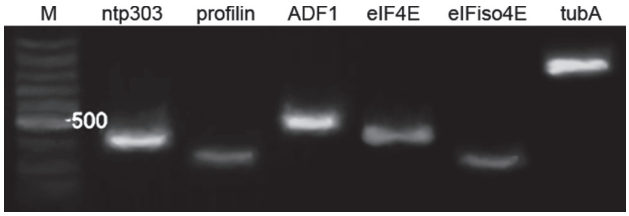

Figure 3. RT-PCR of mRNAs isolated from EPP complexes revealed broad spectra of mRNAs naturally integrated into EPP complexes. mRNAs encoding NTP303, profiling, actin-depolymerizing factor 1 (ADF1), eukaryotic initiation factor (eIF4E) and its isoform (eIFiso4E) and alpha-tubulin A1 (tubA) were analyzed.

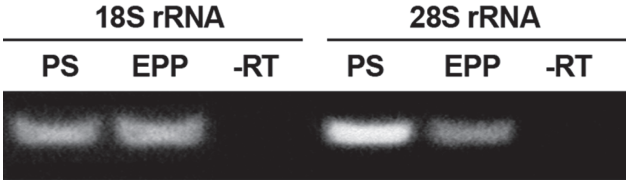

Figure 4. RT-PCR of rRNAs. One microgram of total RNA extracted from EPP complexes (EPP) and polysomes (PS) was reverse transcribed using primers specifically designed against 18S and 28S rRNAs. Negative control reaction was performed without reverse transcriptase (-RT).

comparison with the original protocol (60% sucrose cushion; Figure 2A),[33] no changes in the EPP profile and yet a higher yield were obtained after 3 h and 20 min sedimentation though 30% sucrose under 60 000 rpm (Figure 2C). Alternatively, SEC was employed for the direct isolation of EPP complexes from postmitochondrial supernatant without presedimentation through sucrose cushion. This modification led to approximately 10 times higher increase in yield. Moreover, the purity and homogeneity of mature pollen EPP complexes isolated by various protocols was tested by comparison of their 2-D SDS-PAGE protein profiles. Image analysis (LUCIA G, ver. 4.70) showed no qualitative changes in protein patterns of pollen EPPs (Figure 1A, other data not shown) sedimented through 30% and 60% sucrose cushion compared to those obtained directly by SEC. Hence, the procedure of SEC-based direct EPP purification from the postmitochondrial supernatant without ultracentrifugation step was successfully used for further biochemical analyses to increase the yield of EPP fraction (Figure 2B).

3.3. EPPs Contain Ribosomal Subunits. When the column chromatography revealed the supramolecular character of EPPs, electron microscopy was applied to provide more information about their size and structure. Samples for electron microscopy were prepared from 4-h pollen tubes, in which the store of EPPs was still sufficiently high (Figure 1C). Ultrastructural study displayed the EPP pellet as a heavy ribonucleoprotein complex. Its structure contained sets of heterogeneous particles, ranging from 20 to 60 nm in diameter, rather than uniform components (Supplementary Figure 1 in Supporting Information). Considering their size, they were considered as ribosomal subunits.

To confirm this hypothesis, presence of both major ribosomal rRNAs in EPP complexes was verified by RT-PCR. Total RNA isolated from polysomes and SEC-purified EPPs was reverse-transcribed using primers specifically designed against tobacco 18S and 28S rRNAs. After reverse transcription, complementary rDNA fragments corresponding to both 18S and 28S rRNAs were PCR-amplified from polysomal and EPP fractions (Figure 4). To exclude the amplification of genomic rDNA, total RNA extracted from EPPs was used as a negative control in PCR reaction without preceding reverse transcription. The ultimate proof was obtained at the proteomic level. LC-MALDI MS analyses identified 13 ribosomal proteins from both small (3 proteins) and large (10 proteins) subunits (Table 1, Supplementary Table 2 in Supporting Information, for details see below).

3.4. EPPs Are Associated with Cytoskeleton. The cytoskeleton has repeatedly been found in association with mRNP particles and various components of translational apparatus.[20,21,28] This led us to analyze the potential presence of actin and tubulin in EPPs and polysomes. EPPs were isolated in HSEP buffer, whereas polysomal fraction was extracted with LS buffer.[33] The application of LS buffer preserved polysomal structures but also retained EPP complexes in the polysomal fraction. The proportion of EPPs in polysomal pellet was much smaller than that of EDTA/puromycin-sensitive polysomes. The quantity of proteins comprised in EPPs in 4-h pollen tubes represented about $^{1}/_{10}$ of the whole polysomal pellet.

In the EPP fraction, a weak fluorescence was detected in the stage 3 of immature pollen when the formation of EPP complexes is initiated,[33] but a markedly stronger signal was observed in the mature pollen grains and pollen tubes (Supplementary Figure 2A,B in Supporting Information). This finding clearly indicated high levels of actin associated with the EPP fraction in later stages of pollen development. In the polysomal fraction, no signal was observed in the stage 3 of immature pollen. Actin signal in polysomal pellet from mature pollen and pollen tubes was stronger and comparable to that in the EPP fraction of appropriate developmental stages. In all experiments, images had typical nongranular uniform signal throughout all particles.

The distribution of tubulin followed a similar pattern as that of actin (Supplementary Figure 2C,D in Supporting Information). Tubulin fluorescence in the EPP fraction in immature pollen at the developmental stage 3 was slightly higher in comparison to the autofluorescence of a control sample. However, this result still supported positive immunofluorescence. The strong signal in the EPP fraction of mature pollen grain and pollen tube clearly demonstrated the abundance of tubulin in these stages. On the contrary, the polysomal fraction in immature pollen at the stage 3 showed no significant fluorescence. Nevertheless, tubulin was highly detected in polysomal pellets in mature pollen and pollen tubes with signal intensity similar to that of the EPP fraction.

To eliminate the effect of autofluorescence, the presence of cytoskeletal proteins was verified by Western blot immunodetection within proteins isolated from SEC fractions (Figure 5). It revealed a similar pattern of distribution of both cytoskeletal proteins in the EPP and polysomal fractions as did the immunofluorescence. Compared to polysomes, actin is likely to be a preferable component of EPP complexes in course of EPP formation in immature pollen grains as well as during the pollen tube growth.

These results were confirmed and further extended by proteomic analyses that led to the identification of α-tubulin, profilin-3 and eight actin clones associated with EPPs (Table 1, Supplementary Table 2 in Supporting Informaiton, for details see below).

3.5. EPPs Contain Different mRNAs. EPPs were previously shown to contain translationally repressed pollen-specific

Table 1. Proteins Identified by Mass Spectrometry Analyses in EPPs Isolated from Mature Pollen and 4-h Pollen Tubes[a]

ID	protein name	accession	MW	p*I*	total ion score	peptide count pollen	peptide count tubes	EPP excl.	EPP incl.	function	process	compartment
1	Heat shock protein 70−3 [*Nicotiana tabacum*]	gil38325815	71341	5.14	2014	24	11	Y	Y	Chaperone	Protein fate	Cytosol
2	Adenosylhomocysteinase (*S*-adenosyl-L-homocysteine hydrolase)	gil78102508	53640	5.51	1528	22	9	Y	Y	AA metabolism	Metabolism	Cytosol
3	ATP synthase F1 subunit 1 [*Nicotiana tabacum*]	gil57013987	55476	5.84	1278	18	8	Y	Y	ATP synthesis	Energy metabolism	Mitochondria
4	Actin	gil461465	41940	5.46	683	13	11	Y	Y	Cytoskeleton	Cytoskeleton	Cytosol
5	Luminal-binding protein 5 precursor (BiP 5)	gil729623	73870	5.08	1217	19	5	Y	Y	Chaperone, ERER	Protein fate	Cytosol
6	Vacuolar H^+-ATPase B subunit [*Nicotiana tabacum*]	gil6715512	53975	5.11	719	10	12	Y	Y	Ion transport	Transport	Cytosol
7	Elongation factor-1 alpha [*Nicotiana tabacum*]	gil1864017	49633	9.2	556	11	9	Y	Y	Translation elongation factor	Translation	Cytosol - ribosome
8	Putative UDP-glucose dehydrogenase 2 [*Nicotiana tabacum*]	gil48093459	58943	5.92	671	14	6	Y	Y	Saccharide metabolism	Metabolism	Cytosol
9	Actin-54	gil3219762	37521	5.66	578	11	8	Y	Y	Cytoskeleton	Cytoskeleton	Cytosol
10	Heat shock protein 90 [*Nicotiana tabacum*]	gil46093890	80358	4.95	1093	15	4	Y	Y	Chaperone	Protein fate	Cytosol
11	Luminal-binding protein 4 precursor (BiP 4)	gil729620	73705	5.07	1255	19		N	Y	Chaperone, ER	Protein fate	Cytosol
12	Ribosomal protein L3A [*Nicotiana tabacum*]	gil37625023	44823	10.08	447	8	9	Y	Y	Ribosomal protein	Translation	Cytosol - ribosome
13	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	gil548533	61257	5.98	780	12	3	Y	Y	Glycolysis	Metabolism	Cytosol
14	Ribosomal protein L3B [*Nicotiana tabacum*]	gil37625025	47502	10	376	6	8	Y	Y	Ribosomal protein	Translation	Cytosol - ribosome
15	Ferritin-1, chloroplast precursor (NtFer1)	gil29839371	28464	5.7	332	7	7	Y	Y	Iron storage	Metabolism	Chloroplast
16	Phosphoenolpyruvate carboxylase (PEPCase) (PEPC)	gil115610	110665	5.61	448	2	12	Y	Y	CO_2 fixation	Metabolism	Cytosol
17	Eukaryotic initiation factor 4A-8 (ATP-dependent RNA helicase eIF4A-8)	gil1170508	47143	5.57	307	11	3	Y	Y	Translation initiation factor	Translation	Cytosol - ribosome
18	Aldehyde dehydrogenase (NAD+) [*Nicotiana tabacum*]	gil1743354	59698	6.9	563	9	4	Y	Y	Alcohol metabolism	Energy metabolism	Mitochondria
19	Glyceraldehyde-3-phosphate dehydrogenase [*Nicotiana tabacum*]	gil4539543	36816	7.7	299	9	4	Y	Y	Glycolysis	Metabolism	Cytosol
20	Poly(A)-binding protein [*Nicotiana tabacum*]	gil7673355	71339	7.6	589	10	3	Y	Y	RNA-binding protein	Translation	Cytosol - ribosome
21	Acetyl-CoA carboxylase (CAC), biotin carboxylase subunit	gil870726	58863	6.66	469	8	4	Y	Y	Fatty acid synthesis	Metabolism	Cytosol
22	Eukaryotic initiation factor 4A-13 (ATP-dependent RNA helicase eIF4A-13)	gil2500519	40475	5.17	228	8	4	Y	Y	Translation initiation factor	Translation	Cytosol - ribosome
23	Alcohol dehydrogenase [*Nicotiana tabacum*]	gil551257	41981	6.64	409	10	2	Y	Y	Alcohol metabolism	Energy metabolism	Cytosol
24	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	gil120676	35682	6.14	475	9	2	Y	Y	Glycolysis	Metabolism	Cytosol
25	Pyruvate decarboxylase isozyme 2 (PDC)	gil1706330	68211	5.65	648	11		N	Y	Acetyl CoA biosynthesis, glycolysis	Metabolism	Cytosol
26	Proteasome subunit alpha type-6 (20S proteasome alpha subunit A)	gil12229948	27400	5.92	201	4	6	Y	Y	Protein degradation	Protein fate	Cytosol - proteasome
27	Phosphoglycerate kinase, cytosolic	gil2499498	42338	5.69	421	9	1	Y	Y	Glycolysis	Metabolism	Cytosol
28	Actin-93	gil3219760	37274	5.82	485	10		N	Y	Cytoskeleton	Cytoskeleton	Cytosol
29	Elongation factor 2 [*Nicotiana tabacum*]	gil10798636	16323	8.07	400	6	3	Y	Y	Translation elongation factor	Translation	Cytosol - ribosome
30	Actin-103	gil3219765	37314	5.33	486	9		N	Y	Cytoskeleton	Cytoskeleton	Cytosol
31	Eukaryotic initiation factor 4A-9 (ATP-dependent RNA helicase eIF4A-9)	gil2500517	47081	5.54	250	9		N	Y	Translation initiation factor	Translation	Cytosol - ribosome
32	Adenosine kinase isoform 1T [*Nicotiana tabacum*]	gil51949796	37799	5.13	205	5	3	Y	Y	Nucleoside kinase	Metabolism	Cytosol
33	Actin (clone Tac9) [*Nicotiana tabacum*]	gil7441422	22818	5.29	190	5	3	Y	Y	Cytoskeleton	Cytoskeleton	Cytosol
34	60S ribosomal protein L13 (Clone 6.2.1)	gil1350664	23434	11.4	201	3	5	Y	Y	Ribosomal protein	Translation	Cytosol - ribosome
35	Alpha-tubulin [*Nicotiana tabacum*]	gil17402469	50388	4.89	225	6	2	Y	Y	Cytoskeleton	Cytoskeleton	Cytosol
36	Isocitrate dehydrogenase (NAD+) [*Nicotiana tabacum*]	gil3021506	40598	7.14	275	7	1	Y	Y	Krebs cycle	Metabolism	Mitochondria
37	Fructokinase [*Nicotiana tabacum*]	gil23978579	17993	5.61	164	5	3	Y	Y	Starch synthesis	Metabolism	Cytosol
38	Actin [*Nicotiana tabacum*]	gil50058115	41885	5.31	458		8	N	Y	Cytoskeleton	Cytoskeleton	Cytosol
39	S-adenosyl-L-methionine synthetase [*Nicotiana tabacum*]	gil38261499	43042	5.74	405		8	N	Y	SAM synthesis	Signaling, methylation	Cytosol
40	*S*-adenosylmethionine synthase [*Nicotiana tabacum*]	gil33340517	42970	5.82	276	8		N	Y	SAM synthesis	Signaling, methylation	Cytosol

Table 1. Continued

ID	protein name	accession	MW	p*I*	total ion score	peptide count pollen	peptide count tubes	EPP excl.	EPP incl.	function	process	compartment
41	Glutamine synthetase [*Nicotiana tabacum*]	gi|1419094	39380	5.38	201	4	3	Y	Y	AA biosynthesis, nitrate assimilation	Metabolism	Cytosol
42	60S ribosomal protein L8 (60S ribosomal protein L2)	gi|132849	28437	10.82	208	2	5	Y	Y	Ribosomal protein	Translation	Cytosol - ribosome
43	Elongation factor 2 [*Nicotiana tabacum*]	gi|10798634	20423	9.11	192	4	3	Y	Y	Translation elongation factor	Translation	Cytosol - ribosome
44	Glutamine synthetase [*Nicotiana tabacum*]	gi|1419092	39198	5.72	200	5	2	Y	Y	AA biosynthesis, nitrate assimilation	Metabolism	Cytosol
45	Cytosolic aconitase [*Nicotiana tabacum*]	gi|11066033	98692	5.88	280	5	2	Y	Y	Krebs cycle, Fe homeostasis, RNA-binding	Translation, Metabolism	Cytosol, mitochondria
46	Pyruvate kinase, cytosolic isozyme (PK)	gi|2497543	55839	6.4	482	6	1	Y	Y	Glycolysis	Metabolism	Cytosol
47	Heat shock protein 70 [*Nicotiana tabacum*]	gi|19878	63313	5.21	635	7		N	Y	Chaperone	Protein fate	Cytosol
48	Luminal-binding protein 2 (BiP 2)	gi|729618	32354	4.53	467	7		N	Y	Chaperone, ER	Protein fate	Cytosol
49	Poly(A)-binding protein [*Nicotiana tabacum*]	gi|7673359	52706	8.3	418	7		N	Y	RNA-binding protein	Translation	Cytosol - ribosome
50	Elongation factor 1-alpha [*Nicotiana tabacum*]	gi|4731316	49547	9.35	327	7		N	Y	Translation elongation factor	Translation	Cytosol - ribosome
51	Heat shock protein 70−1 [*Nicotiana tabacum*]	gi|38325811	71195	5.02	320	4	2	Y	Y	Chaperone	Protein fate	Cytosol
52	Histone H3 [*Arabidopsis thaliana*]	gi|15232146	15316	11.29	179	2	4	Y	Y	DNA-binding	Transcription	Nucleus
53	Phragmoplastin [*Nicotiana tabacum*]	gi|5931765	68489	7.62	155	4	2	Y	Y	Cell plate formation, GTPase, protein binding	Cytoskeleton, protein fate	Nucleus
54	NAD-malate dehydrogenase [*Nicotiana tabacum*]	gi|5123836	43679	8.03	165	5	1	Y	Y	Krebs cycle, lipid metabolism	Metabolism	Mitochondria, cytosol
55	Eukaryotic translation initiation factor 3 subunit 9 (eIF-3-eta)	gi|6685536	82814	4.93	96	3	2	Y	Y	Translation initiation factor	Translation	Cytosol - ribosome
56	Heat shock protein 82	gi|547684	58270	5.07	343	5		N	Y	Chaperone	Protein fate	Cytosol
57	Putative mitochondrial malate dehydrogenase [*Nicotiana tabacum*]	gi|48375044	22195	7.64	196	5		N	Y	Krebs cycle, lipid metabolism	Metabolism	Mitochondria, cytosol
58	60S ribosomal protein L23a (L25)	gi|585876	17271	10.18	192	5		N	Y	Ribosomal protein	Translation	Cytosol - ribosome
59	Malate dehydrogenase [*Nicotiana tabacum*]	gi|10798652	35727	5.91	150	5		N	Y	Krebs cycle, lipid metabolism	Metabolism	Mitochondria, cytosol
60	Putative alpha7 proteasome subunit [*Nicotiana tabacum*]	gi|14594925	27466	6.11	104	2	2	Y	Y	Protein degradation	Protein fate	Cytosol - proteasome
61	Ribosomal protein L11-like [*Nicotiana tabacum*]	gi|10799832	20932	9.94	153	3	1	Y	Y	Ribosomal protein	Translation	Cytosol - ribosome
62	Profilin-3	gi|14423866	14606	5.04	95	2	2	Y	Y	Cytoskeleton	Cytoskeleton	Cytosol
63	Ribosomal protein S6 [*Nicotiana tabacum*]	gi|20022	21942	10.76	110	2	2	Y	Y	Ribosomal protein	Translation	Cytosol - ribosome
64	H2A histone [*Nicotiana tabacum*]	gi|27529852	15457	10.6	159	1	3	Y	Y	DNA-binding	Transcription	Nucleus
65	60S ribosomal protein L10 (QM protein homologue)	gi|2500355	16849	10.8	65	2	2	Y	Y	Ribosomal protein	Translation	Cytosol - ribosome
66	Putative beta4 proteasome subunit [*Nicotiana tabacum*]	gi|14594929	14590	6.4	174	1	3	Y	Y	Protein degradation	Protein fate	Cytosol - proteasome
67	Ras-related protein Rab11A	gi|3024506	24199	8.37	49	2	2	Y	Y	GTPase	Transport	Cytosol
68	Histone H3.2 [*Arabidopsis thaliana*]	gi|15236103	15454	11.15	175		4	N	Y	DNA-binding	Transcription	Nucleus
69	60S ribosomal protein L2 [*Nicotiana tabacum*]	gi|9230281	18578	10.87	151		4	N	Y	Ribosomal protein	Translation	Cytosol - ribosome
70	GDP dissociation inhibitor [*Nicotiana tabacum*]	gi|2501850	50127	5.44	206	4		N	Y	Protein transport	Protein transport	Cytosol
71	Sucrose-6-phosphate phosphatase [*Nicotiana tabacum*]	gi|57018995	48210	5.93	152	4		N	Y	Phosphatase, sucrose transport	Metabolism	Cytosol
72	Histone H2B	gi|7387729	15947	10.13	101	1	2	Y	Y	DNA-binding	Transcription	Nucleus
73	60S ribosomal protein L34	gi|730558	13801	11.6	83	2	1	Y	Y	Ribosomal protein	Translation	Cytosol - ribosome
74	Putative UDP-glucuronate decarboxylase 3 [*Nicotiana tabacum*]	gi|48093465	50012	9.37	65	1	2	Y	Y	Sugar metabolism	Metabolism	Cytosol
75	Ras-related protein RAB8 −1 [*Nicotiana tabacum*]	gi|18447913	24130	7.66	45	2	1	Y	Y	GTPase	Protein transport	Cytosol
76	NADH dehydrogenase subunit 7 [*Nicotiana tabacum*]	gi|57014017	44784	6.95	153		3	N	Y	Respiration, electron transport	Energy metabolism	Mitochondria
77	Adenosine kinase isoform 1S [*Nicotiana tabacum*]	gi|51949800	37820	5.07	152		3	N	Y	Nucleoside kinase	Metabolism	Cytosol

Table 1. Continued

ID	protein name	accession	MW	p*I*	total ion score	peptide count pollen	peptide count tubes	EPP excl.	EPP incl.	function	process	compartment
78	Putative pyridoxine biosynthesis protein isoform A [*Nicotiana tabacum*]	gi|46399269	33353	5.93	127		3	N	Y	Protein homodimerization	Protein fate, Metabolism, Stress	Cytosol
79	Putative beta5 proteasome subunit [*Nicotiana tabacum*]	gi|14594931	18712	9.21	118		3	N	Y	Protein degradation	Protein fate	Cytosol - proteasome
80	NADH dehydrogenase subunit 9 [*Nicotiana tabacum*]	gi|57014008	22738	6.44	100		3	N	Y	Respiration, electron transport	Energy metabolism	Mitochondria
81	UDP-glucose pyrophosphory lase [*Nicotiana tabacum*]	gi|17402533	41229	7.12	170	3		N	Y	Sugar metabolism	Metabolism	Cytosol
82	NTP101 [*Nicotiana tabacum*]	gi|37223173	62845	8.85	80		3	N	Y	Pectin metabolism	Cell wall metabolism	Cytosol
83	Adenosine kinase isoform 2S [*Nicotiana tabacum*]	gi|51949802	37929	5.16	155	3		N	Y	Nucleoside kinase	Metabolism	Cytosol
84	*N*-ethylmaleimide sensitive fusion protein [*Nicotiana tabacum*], ATPase	gi|1449179	81355	5.81	152	3		N	Y	Membrane fusion	Transport	Cytosol
85	Elongation factor 2 [*Nicotiana tabacum*]	gi|1841462	20147	5.34	135	3		N	Y	Translation elongation factor	Translation	Cytosol - ribosome
86	Protein kinase 1 [*Nicotiana tabacum*]	gi|38488407	39482	6.51	80	3		N	Y	Protein kinase	Signaling	Cytosol
87	Protein kinase 2 [*Nicotiana tabacum*]	gi|38488409	39902	8.12	80	3		N	Y	Protein kinase	Signaling	Cytosol
88	Enoyl-ACP reductase [*Nicotiana tabacum*]	gi|2204236	41925	8.88	69	3		N	Y	Lipid metabolism	Metabolism	Cytosol
89	Inositol-3-phosphate synthase	gi|14548096	56505	5.53	104	2		N	Y	Inositol-P metabolism, phospholipid synthesis	Signaling	Cytosol
90	GTP-binding nuclear protein Ran-B1	gi|1172836	25498	6.45	83	2		N	Y	Protein import, RNA export	Protein transport	Cytosol, nucleus
91	Putative UDP-glucuronate decarboxylase 4 [*Nicotiana tabacum*]	gi|48093467	46001	9.04	61	2		N	Y	Sugar metabolism	Cell wall metabolism	Cytosol - Golgi membranes
92	Protein phosphatase 2A [*Nicotiana tabacum*]	gi|1568511	66079	5.03	61	2		N	Y	Protein phosphatase	Signaling	Cytosol
93	Cytochrome b-c1 complex subunit Rieske-2, mitochondrial precursor	gi|1717950	29993	8.67	56	2		N	Y	Cytochrome c reduction	Energy metabolism	Mitochondria
94	Cytosolic acetoacetyl-coenzyme A thiolase [*Nicotiana tabacum*]	gi|53854350	41699	6.47	50	2		N	Y	AA metabolism	Metabolism	Cytosol
95	GDP-D-mannose pyrophosphory lase [*Nicotiana tabacum*]	gi|14971013	39889	6.11	49	2		N	Y	ascorbate biosynthesis	Stress, Metabolism	Cytosol
96	Pyruvate dehydrogenase E1 alpha subunit [*Nicotiana tabacum*]	gi|28465347	14807	6.3	38	2		N	Y	Acetyl CoA biosynthesis, glycolysis	Metabolism	Mitochondria
97	ATP synthase CF1 beta subunit [Nicotiana sylvestris]	gi|78102542	53563	5.1	81	1	1	N	Y	ATP synthesis	Energy metabolism	Mitochondria
98	ubiquitin [*Nicotiana tabacum*]	gi|1762935	8710	8.04	70	1	1	N	Y	Protein degradation	Protein fate	Cytosol - proteasome
99	Translationally controlled tumor protein homologue (TCTP)	gi|9979177	18729	4.36	39	1	1	N	Y	MT stabilization	Cytoskeleton, signaling	Cytosol
100	Ferritin-2, chloroplast precursor (NtFer2)	gi|29839345	29316	6.09	37	1	1	N	Y	Iron storage	Metabolism	Chloroplast
101	Catalase 1 [*Nicotiana tabacum*]	gi|2459684	57225	6.86	32	1	1	N	Y	Hydrogen peroxide decomposition	Stress	Cytosol - peroxisome
102	40S ribosomal protein S14	gi|2500443	6996	11.92	28	1	1	N	Y	Ribosomal protein	Translation	Cytosol - ribosome
103	Ethylene forming enzyme (EFE) [*Nicotiana tabacum*]	gi|450357	36364	5.51	31	1	1	N	Y	Oxidoreductase	Stress	Cytosol
104	WREBP-2 [*Nicotiana tabacum*]	gi|4519673	42860	6.3	23	1	1	N	Y	Zinc finger protein	Transcription	Nucleus
105	Calmodulin (CaM)	gi|3121848	16880	4.1	41	1	1	N	Y	Signaling	Signaling	Cytosol
106	Ribosomal protein S4 [*Nicotiana tabacum*]	gi|57014001	41690	10.8	24	1	1	N	Y	Ribosomal protein	Translation	Cytosol - ribosome
107	Putative rac protein [*Nicotiana tabacum*]	gi|27527525	24087	9.39	22	1	1	N	Y	GTPase	Signaling	Cytosol
108	Hypothetical protein NitaMp143 [*Nicotiana tabacum*]	gi|57014009	12308	9.91	20	1	1	N	Y	Unknown	Unknown	Unknown
109	Avr9/Cf-9 rapidly elicited protein 74 [*Nicotiana tabacum*]	gi|30013683	50431	8.7	21	1	1	N	Y	Defense response	Stress	Cytosol
110	Membrane-associated salt-inducible protein	gi|473874	49756	8.49	21	1	1	N	Y	Unknown	Unknown	Cytosol
111	Arginine decarboxylase [*Nicotiana tabacum*]	gi|40645472	80178	4.99	22	1	1	N	Y	AA metabolism	Metabolism	Cytosol
112	Eukaryotic translation initiation factor 3 subunit 10 (eIF-3-theta)	gi|6685538	111944	9.4	20	1	1	N	Y	Translation initiation factor	Translation	Cytosol - ribosome

Table 1. Continued

ID	protein name	accession	MW	p*I*	total ion score	peptide count pollen	peptide count tubes	EPP excl.	EPP incl.	function	process	compartment
113	Ferredoxin--nitrite reductase [*Nicotiana tabacum*]	gil19893	65680	5.89	20	1	1	N	Y	Electron transport	Energy metabolism	Chloroplast
114	Avr9/Cf-9 rapidly elicited protein 216 [*Nicotiana tabacum*]	gil30013673	47661	8.47	18	1	1	N	Y	Defense response	Stress	Cytosol

[a] Only proteins reliably identified with more than one peptide are shown. Proteins associated with EPPs identified by both exclusive ("EPP excl.") and inclusive ("EPP incl.") approach are marked (Y = yes; N = no; for detailed description see text). For each protein, elementary data, "accession", molecular weight ("MW"), p*I* value ("p*I*"), "total ion score", peptide counts in EPPs isolated from mature pollen ("peptide count pollen") and 4-h pollen tubes ("peptide count tubes") are given. The "total ion score" column shows the higher value from both experiments. Complete data for all proteins identified in both experiments are presented in Supplementary Table 2 in Supporting Information.

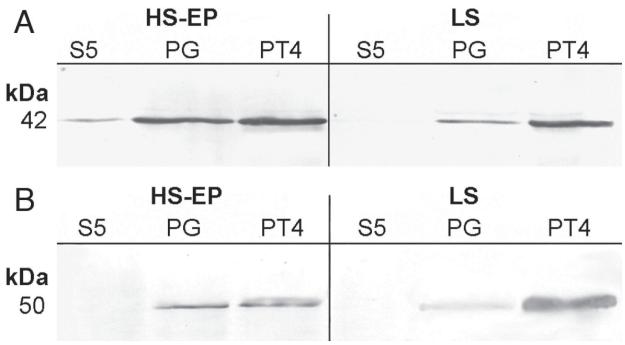

Figure 5. 1-D immunoblot of cytoskeletal proteins present within the EPP fraction. Actin (A) and tubulin (B) were immunodetected in midbicellular pollen stage (S5), mature pollen (PG) and in 4 h pollen tubes (PT4) isolated with LS and HS-EP buffer.

ntp303 mRNA.[33] To examine whether they also contained other transcripts, RT-PCR analysis was performed. Several tobacco pollen-specific genes were tested including *profilin*,[39] *actin depolymerizing factor 1* (ADF1),[40] *eukaryotic translation initiation factors eIF4E*, *eIFiso4E*[41] and α-*tubulin A1* (accession number AJ421411; D. Brevario, direct submission). RT-PCR analysis revealed that all tested transcripts were present in the EPP fractions isolated by ultracentrifugation as well as directly from SEC (Figure 3).

3.6. EPPs Represent Not only an mRNA Storage Compartment, but a Complex Machinery Devoted to the mRNA Transport and Protein Synthesis, Processing, and Precise Localization. Proteins contained in the EPP ribonucleoprotein particles isolated by ultracentrifugation from mature pollen were separated by 1-D SDS-PAGE and then subjected to LC-MALDI MS. We identified 168 individual proteins. Of them, 82 proteins identified by more than one peptide were summarized in Table 1. The remaining 86 proteins identified by one peptide only were considered less convincing and are summarized in Supplementary Table 2 in Supporting Information. Functional categorization of 82 positively identified proteins revealed several classes (Figure 6A). Thirty-four proteins (41.4%) were involved in the RNA and protein metabolism, namely, protein synthesis (21), protein fate (10), and protein transport (3). A function in the regulation of RNA localization and translation can be also assigned to eight cytoskeletal proteins and three protein kinases and phosphatases. Interestingly, proteomic analysis revealed further 25 EPP proteins (30.5%) involved in the general metabolism and one protein (1.2%) involved in the synthesis of cell wall precursors. Among them, a substantial fraction was made up of enzymes catalyzing five particular glycolytic reactions and five reactions of the Krebs cycle. Considering the subcellular localization of identified proteins, 74 of them (90%) were localized in the cytosol. They were supplemented with two nuclear proteins, five mitochondrial and one plastid-localized protein. Of the cytosolic proteins, a substantial fraction of 20 proteins was associated with ribosomes and/or other ribonucleoproteins and two proteins represented proteasome subunits.

Since we were aware of the extremely compact organization of the mature pollen cytoplasm and thus a higher risk of contamination of purified EPP complexes, we performed the same proteomic analysis on EPPs isolated from pollen tubes cultivated *in vitro* for 4 h. These pollen tubes are fully hydrated and actively growing, however, still before the onset of pollen mitosis II. The results of proteomic analyses were similar to those of mature pollen. We identified 121 individual proteins. Of them, 58 proteins identified by more than one peptide are summarized in Table 1. The 63 remaining proteins identified by one peptide only were considered less reliable and are summarized in Supplementary Table 2 in Supporting Information. Proteins identified in mature pollen and 4-h pollen tubes are indicated in both tables. Accordingly, the functional categorization of EPP proteins isolated from pollen tubes is similar to that of mature pollen (Figure 6B). Twenty-four proteins (41.3%) were involved in protein synthesis (15) and protein fate (9). The representation of cytoskeletal proteins (6) and a protein involved in cell wall metabolism (1) was similar as well as the proportion of those involved in general metabolism (14, i.e., 24%). A higher number of nuclear (5), mitochondrial (4) and plastid-localized (1) proteins resulted in lower fraction of cytosolic proteins (48, i.e., 83%). Of the cytosolic proteins, 14 were associated with ribosomes and/or other ribonucleoproteins and four proteins represented proteasome subunits.

For the combination of both data sets, two alternative approaches were used, exclusive and inclusive. The inclusive approach grouped proteins with at least two peptide counts on aggregate with no strict need for their presence in both data sets. After such treatment, few new proteins appeared in the final list because, in addition, proteins with one peptide count in each data set were included. A more stringent, exclusive approach was applied to subselect only proteins present in both data sets by at least one peptide, but reliably present in at least one data set with more than one peptide. The application of both approaches had a significant effect on the number of identified proteins. The inclusive method resulted in the identification of 114 EPP associated proteins. On the contrary, the application of the exclusive approach reduced this number to 55 individual polypeptides. However, the functional categorization of both data sets (in fact a larger data set and its subset) did not differ considerably (Figure 6C,D); the exclusive treatment led to the elimination of proteins involved in signaling, cell wall metabolism, stress response and of unknown function.

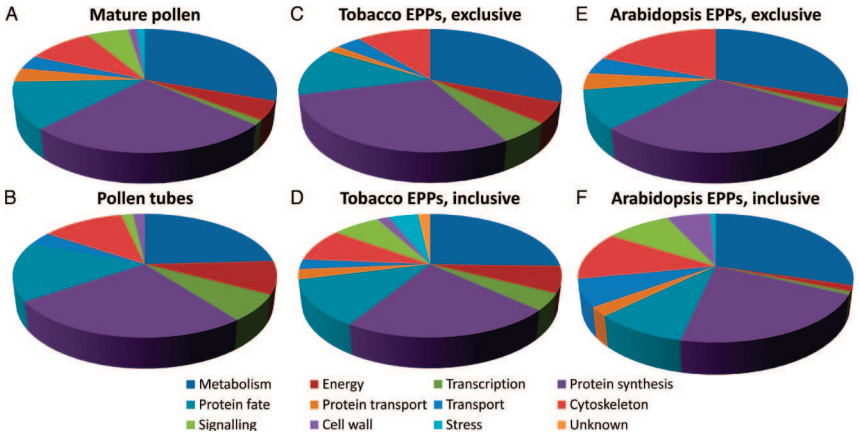

Figure 6. Functional categorization of EPP proteins isolated from tobacco mature pollen (A) and 4-h pollen tubes (B). Combination of both data sets after application of exclusive (C) and inclusive (D) approach is compared. Functional categorization of analogous exclusively (E) and inclusively identified (F) data sets from *Arabidopsis* pollen.

Taken together, the exclusively identified proteins are likely to comprise core EPP complexes or proteins associated within longer residence, whereas the inclusive method highlighted transiently associated proteins, possibly with regulatory and/or targeting function(s), possibly during the activation phase. Moreover, a number of transiently associated proteins together with presumed dynamics of EPPs composition, especially during early phases of progamic phase, makes the inclusive set of proteins worth consideration and further research.

With the exception of actin depolymerising factor 1 (ADF1), proteins encoded by all other transcripts found within EPPs were also identified by LC-MALDI MS. This observation suggested that a fraction of proteins detected within EPP complexes can represent translational products of associated transcripts.

3.7. Expression Profiles of *Arabidopsis* Orthologues of Genes Encoding Tobacco EPP Proteins Suggests the Existence and Activity of EPPs in Other Species. The just described complex role of EPPs in tobacco cytoplasmic mRNA and protein metabolism makes them likely to be active in another plant species as well. Another model plant, *Arabidopsis thaliana*, has more advanced tricellular pollen with different pollen tube growth strategy and, accordingly, gene expression. In our previous study, we demonstrated that in *Arabidopsis* the active pollen tube growth is strictly dependent upon protein synthesis, and that pollen germination and tube growth are relatively independent of transcription.[42]

Here, we exploited available *Arabidopsis* proteomic and transcriptomic resources to identify genes presumably comprising structures analogous to EPP complexes. We identified the closest *Arabidopsis* orthologues of all identified *N. tabacum* EPP proteins. There was an apparent overlap with proteins described previously as a part of male gametophyte proteome. *Arabidopsis* orthologues of 44 out of 114 tobacco EPP proteins were identified in at least one *A. thaliana* mature pollen proteome.[43−45] Because of sequence similarity and/or possible functional redundancy, it represented 53 *Arabidopsis* proteins (Table 2), 27 of which were identified by Noir et al.,[44] 25 by Holmes-Davies et al.,[43] and 12 by Sheoran et al.[45] Three published male gametophytic proteomic studies identified a limited number of proteins (267 proteins)[43−45] in relation to the number of genes presumably active in mature pollen and discovered by transcriptomics (4774−5938 genes).[42,46,47] From this perspective, EPP proteins are significantly overrepresented in the known fraction of male gametophytic proteome.

Previously identified proteins were supplemented with more EPP orthologous genes that were not previously found in proteomic studies but have positive expression signal in developing male gametophyte.[42] These analyses led to the identification of 83 *Arabidopsis* genes encoding orthologues of 50 tobacco EPP proteins. The elimination of duplicates resulted in the finalized table summarizing 131 *Arabidopsis* orthologues of 79 tobacco EPP proteins (Table 2). Only for four tobacco mature pollen EPP proteins were no positives identified, mainly because of the absence of orthologues positively expressed in male gametophyte. Not surprisingly, functional classification revealed that both gene sets were similar (Figure 6).

Expression profiles of *Arabidopsis* genes revealed two distinct groups of genes encoding EPP protein orthologues. The first group consisted mainly of ribosomal proteins that were previously shown to be encoded by early gametophytic genes.[42] However, the majority of genes encoding nonribosomal proteins was encoded by late genes. Surprisingly, the expression of many of these genes peaked in the tricellular stage. In *Arabidopsis* transcriptome, genes with this expression profile formed a relatively small cluster (88 genes out of 13 977 active).[42] However, among the orthologues of EPP proteins, this characteristic profile was markedly overrepresented.

4. Discussion

The identification of EPP complexes in immature and mature pollen represented the first direct proof of long time-hypothesized presence of stored mRNP particles in developing tobacco male gametophyte. Moreover, their specific character was demonstrated by *in vitro* translation that presented EPPs as functional units with regulated translational activity.[33] The existence of these complexes explained the apparent discrepancy between the limitation of transcription to the first 3 h of tobacco pollen germination[29] and formation of 4 cm-long pollen tubes demanding massive translation.[48] Nevertheless, exact timing of derepression of mRNAs stored in EPP complexes remained unknown. Rehydration of pollen grains was one of the hypothesized triggers for the activation of translationally silent mRNAs. In such a case, the half-life of stored mRNA would have to be extremely long.

Gradual activation of mRNAs during pollen tube growth represented the possible mode of translational regulation of stored mRNAs. This concept was supported by observed presence of EPP complexes in pollen tubes even after 24 h of cultivation (Figure 1D). Stable qualitative protein spectra of EPP

Table 2. Closest *A. thaliana* Orthologues of Identified Tobacco EPP Proteins[a]

AGI name	protein	score	*E*-value	EPP excl.	EPP incl.	pollen proteome			pollen transcriptome	ID tobacco
						Noir	Holmes	Sheoran		
At1g04410	Malate dehydrogenase, cytosolic	1534	1.8×10^{-158}	N	Y	N	Y	N	N	59
At1g04820	TUA4, tubulin alpha-4 chain	2135	3.8×10^{-222}	Y	Y	N	Y	N	N	35
At1g06700	Serine/threonine protein kinase	1191	4.1×10^{-122}	N	Y	N	N	N	Y	86
At1g07410	AtRABA2b, *Arabidopsis* Rab GTPase homologue A2b	903	1.3×10^{-91}	Y	Y	N	N	N	Y	67
At1g07920	eEF-1-alpha	2277	3.4×10^{-237}	Y	Y	Y	Y	N	N	7, 50
At1g07930	eEF-1-alpha	2277	3.4×10^{-237}	Y	Y	N	Y	N	N	7, 50
At1g09080	BIP3, luminal binding protein	2452	9.6×10^{-256}	Y	Y	N	N	Y	N	5, 11, 48
At1g09780	2,3-biphosphoglycerate-independent phosphoglycerate mutase	2309	1.4×10^{-240}	Y	Y	N	Y	N	N	13
At1g13320u	PP2AA3, protein phosphatase 2A subunit A3	2639	1.5×10^{-275}	N	Y	N	N	N	Y	92
At1g13440	GAPC-2, glyceraldehyde-3-phosphate dehydrogenase C-2	1450	1.5×10^{-149}	Y	Y	N	Y	N	N	19, 24
At1g14320	60S ribosomal protein L10	613	7.2×10^{-61}	Y	Y	N	N	N	Y	65
At1g14830	ADL1C, *Arabidopsis* dynamin-like protein 1C, GTPase	2186	1.5×10^{-227}	Y	Y	N	N	N	Y	53
At1g19890	ATMGH3, male-gamete-specific histone H3	524	2.0×10^{-51}	Y	Y	N	N	N	Y	52, 68
At1g20260	Vacuolar ATP synthase subunit B3	2205	1.4×10^{-229}	Y	Y	N	Y	N	N	6
At1g22760	PAB3, poly(A)-binding protein 3	1281	2.1×10^{-156}	Y	Y	N	N	N	Y	20, 49
At1g23800	Mitochondrial aldehyde dehydrogenase; nuclear gene for mitochondrial product	2117	3.0×10^{-220}	Y	Y	N	N	N	Y	18
At1g24180	Pyruvate dehydrogenase E1 alpha subunit	682	3.5×10^{-68}	N	Y	N	N	N	Y	96
At1g25490	PP2AA1, protein phosphatase 2A subunit A1	2583	1.3×10^{-269}	N	Y	N	N	N	Y	92
At1g26880	60S ribosomal protein L34A	471	8.1×10^{-46}	Y	Y	N	N	N	Y	73
At1g26910	60S ribosomal protein L10	619	1.7×10^{-61}	Y	Y	N	N	N	Y	65
At1g43170	*Arabidopsis* ribosomal protein ARP1	1771	1.4×10^{-183}	Y	Y	N	N	N	Y	12
At1g48470	GLN1.5, glutamine synthetase 1.5	1500	7.3×10^{-155}	Y	Y	N	N	N	Y	44
At1g49240	ACT8, actin 8	1805	3.5×10^{-187}	Y	Y	N	N	N	Y	4, 9, 28, 30, 33
At1g50010	TUA2, tubulin alpha-2,4	2135	3.8×10^{-222}	Y	Y	Y	N	N	N	35
At1g53240	Malate dehydrogenase (NAD), mitochondrial	932	1.1×10^{-94}	Y	Y	N	N	Y	N	54
At1g53310	ATPPC1, phosphoenolpyruv ate carboxylase 1	4367	0.	Y	Y	N	N	N	Y	16
At1g55560	SKS14 (SKU5 Similar 14), copper ion binding/oxidoreductase	1955	4.5×10^{-203}	N	Y	N	N	N	Y	82
At1g55570	SKS12 (SKU5 Similar 12), copper ion binding/oxidoreductase	1973	5.5×10^{-205}	N	Y	N	N	N	Y	82
At1g56075	eEF2, eukaryotic elongation factor 2	692	3.1×10^{-69}	Y	Y	Y	N	N	N	29, 43, 85
At1g59900	Pyruvate dehydrogenase complex (PDC) alpha subunit	677	1.2×10^{-67}	N	Y	N	N	N	Y	96
At1g64740	Alpha-tubulin expressed primarily in stamens and mature pollen	1995	2.6×10^{-207}	Y	Y	N	N	N	Y	35
At1g66580	60S ribosomal protein L10	591	1.5×10^{-58}	Y	Y	N	N	N	Y	65
At1g69620	60S ribosomal protein L34	471	8.1×10^{-46}	Y	Y	N	N	N	Y	73
At1g71770	PAB5, poly(A)-binding protein 5	1218	6.6×10^{-151}	Y	Y	N	N	N	Y	20, 49
At1g79550	PGK, phosphoglycerate kinase, cytosolic	1814	3.9×10^{-188}	Y	Y	Y	N	N	N	27
At2g05710	Aconitate hydratase, cytoplasmic	4150	0.	Y	Y	Y	N	N	N	45
At2g05840	PAA2, 20S proteasome alpha subunit A2	1156	2.1×10^{-118}	Y	Y	N	N	N	Y	26
At2g05990	ENR1, enoyl-ACP reductase	1371	3.4×10^{-141}	N	Y	N	Y	N	N	88
At2g19760	PFN1, profilin 1	458	1.9×10^{-44}	Y	Y	N	N	Y	N	62
At2g19770	PFN5, profilin 5	520	5.2×10^{-51}	Y	Y	N	N	Y	Y	62
At2g22240	Inositol-3-phosphate synthase 2	2388	5.8×10^{-249}	N	Y	N	N	N	Y	89
At2g22780	Peroxisomal NAD-malate dehydrogenase 1	724	1.2×10^{-72}	N	Y	N	N	N	Y	57
At2g27020	PAG1, 20S proteasome alpha subunit G1	1089	2.6×10^{-111}	Y	Y	N	N	N	Y	60

Table 2. Continued

AGI name	protein	score	*E*-value	EPP excl.	EPP incl.	pollen proteome			pollen transcriptome	ID tobacco
						Noir	Holmes	Sheoran		
At2g31390	pfkB-type carbohydrate kinase family protein; probable fructokinase-1	784	5.5×10^{-79}	Y	Y	Y	N	N	N	37
At2g35840	SPP1, sucrose-phosphatase 1	1608	2.6×10^{-166}	N	Y	Y	N	N	N	71
At2g37620	ACT1, actin 1	1847	1.2×10^{-191}	Y	Y	Y	Y	N	N	4, 9, 28, 30, 33
At2g39460	60S ribosomal protein L23	521	4.1×10^{-51}	N	Y	N	N	N	Y	58
At2g39770	GDP-mannose pyrophosphorylase/ mannose-1-pyrophosphatase	1672	4.4×10^{-173}	N	Y	N	N	N	Y	95
At2g41970	Serine/threonine protein kinase	1506	1.7×10^{-155}	N	Y	N	N	N	Y	86
At2g42600	ATPPC2, phosphoenolpyruv ate carboxylase 2	3485	0.	Y	Y	N	N	N	Y	16
At2g42740	60S ribosomal protein L16A	830	7.3×10^{-84}	Y	Y	N	N	N	Y	61
At2g43230	Serine/threonine protein kinase	1160	7.8×10^{-119}	N	Y	N	N	N	Y	86
At2g44590	ADL1D, *Arabidopsis* dynamin-like protein 1D, GTPase	2064	1.3×10^{-214}	Y	Y	N	N	N	Y	53
At2g47650	UXS4, UDP-XYLOSE SYNTHASE 4	1518	9.1×10^{-157}	N	Y	N	Y	Y	N	91
At3g03250	UGP, UDP-glucose pyrophosphorylase	1675	2.1×10^{-173}	N	Y	N	Y	N	N	81
At3g04120	GAPC-1, glyceraldehyde-3-phosphate dehydrogenase C-1	1451	1.1×10^{-149}	Y	Y	N	Y	N	N	19, 24
At3g08590	2,3-biphosphoglycerate-independent phosphoglycerate mutase	2342	4.4×10^{-244}	Y	Y	Y	N	N	N	13
At3g09810	Isocitrate dehydrogenase, putative	1420	2.2×10^{-146}	Y	Y	N	N	N	Y	36
At3g09820	ADK1, adenosine kinase 1	1526	1.3×10^{-157}	Y	Y	Y	N	N	N	83
At3g09820	ADK 1, adenosine kinase 1	1526	1.3×10^{-157}	Y	Y	Y	N	N	Y	32
At3g13390	SKS11 (SKU5 Similar 11); copper ion binding/oxidoreductase	1998	1.2×10^{-207}	N	Y	N	Y	N	Y	82
At3g13400	SKS13 (SKU5 Similar 13); copper ion binding/oxidoreductase	1958	2.1×10^{-203}	N	Y	N	N	N	Y	82
At3g13920	eIF4A, eukaryotic initiation factor 4Aelongation factor 2	1948	2.5×10^{-202}	Y	Y	Y	N	N	N	17, 22, 31
At3g14940	ATPPC3, phosphoenolpyruv ate carboxylase 3	4371	0.	Y	Y	N	N	N	Y	16
At3g15020	Malate dehydrogenase (NAD), mitochondrial	934	7.0×10^{-95}	Y	Y	N	N	Y	N	54
At3g17390	MAT4, methionine adenosyltransferase 4	1755	7.0×10^{-182}	N	Y	N	N	N	Y	40
At3g17820	GLN1.3, glutamine synthetase 1.3	1585	7.2×10^{-164}	Y	Y	N	N	N	Y	44
At3g18780	ACT2, actin 2	1801	9.3×10^{-187}	Y	Y	N	Y	N	N	4, 9, 28, 30, 33
At3g23810	Adenosylhomocysteinase	2211	3.3×10^{-230}	Y	Y	N	N	N	Y	2
At3g25800	PP2AA2, protein phosphatase 2A subunit A2	2723	1.8×10^{-284}	N	Y	N	N	N	Y	92
At3g28900	60S ribosomal protein L34C	446	3.6×10^{-43}	Y	Y	N	N	N	Y	73
At3g46060	ATRAB8A, ras-related protein RAB8A	852	3.4×10^{-86}	Y	Y	N	N	N	Y	67
At3g46440	UXS5, UDP-XYLOSE SYNTHASE 5	1207	5.7×10^{-126}	N	Y	N	N	N	Y	91
At3g46830	AtRABA2c, *Arabidopsis* Rab GTPase homologue A2c	888	5.2×10^{-90}	Y	Y	N	N	N	Y	67
At3g47520	Malate dehydrogenase (NAD), mitochondrial	682	3.5×10^{-68}	Y	Y	N	Y	N	N	54
At3g48960g	60S ribosomal protein L13C	601	1.4×10^{-59}	Y	Y	N	N	N	Y	34
At3g49010	60S ribosomal protein L13	695	1.5×10^{-69}	Y	Y	N	N	N	Y	34
At3g51190	60S ribosomal protein L8B	1083	1.1×10^{-110}	Y	Y	N	N	N	Y	42, 69
At3g53520	UXS1, UDP-XYLOSE SYNTHASE 1	1747	4.9×10^{-181}	N	Y	N	N	N	Y	91
At3g53610	ATRAB8B, ras-related protein RAB8B	858	7.9×10^{-87}	Y	Y	N	N	N	Y	67
At3g53750	ACT3, actin 3	1847	1.2×10^{-191}	Y	Y	Y	N	Y	N	4, 9, 28, 30, 33
At3g55280	60S ribosomal protein L23A	517	1.1×10^{-50}	N	Y	N	N	N	Y	58
At3g56090	ATFER3, ferritin 3	811	7.5×10^{-82}	Y	Y	N	N	N	Y	15
At3g58700	60S ribosomal protein L11B	830	7.3×10^{-84}	Y	Y	N	N	N	Y	61
At3g59350	Serine/threonine protein kinase	1188	8.5×10^{-122}	N	Y	N	N	N	Y	86

Table 2. Continued

AGI name	protein	score	*E*-value	EPP excl.	EPP incl.	pollen proteome			pollen transcriptome	ID tobacco
						Noir	Holmes	Sheoran		
At3g59480	pfkB-type carbohydrate kinase family protein	800	1.1×10^{-80}	Y	Y	Y	N	N	N	37
At3g59920	ATGDI2, RAB GDP dissociation inhibitor 2	1998	1.2×10^{-207}	N	Y	N	Y	N	N	70
At3g61760	ADL1B, *Arabidopsis* dynamin-like protein 1B, GTPase	2599	2.6×10^{-271}	Y	Y	N	N	N	Y	53
At4g01850	MAT2/SAM2, methionine adenosyltransferase 2/S-adenosylmethionine synthetase 2	1735	9.2×10^{-180}	N	Y	Y	N	N	Y	40
At4g04910	NSF, N-ethylmaleimide sensitive fusion protein, ATPase	3090	0.	N	Y	N	N	N	Y	84
At4g10260	pfkB-type carbohydrate kinase family protein	678	9.4×10^{-68}	Y	Y	Y	Y	N	N	37
At4g13940	Adenosylhomocysteinase	2216	9.8×10^{-231}	Y	Y	Y	N	N	N	2
At4g26390	Pyruvate kinase, putative	2066	7.7×10^{-215}	Y	Y	N	N	N	Y	46
At4g26970	Aconitate hydratase, cytoplasmic	3865	0.	Y	Y	Y	N	N	N	45
At4g29340	PFN4, profilin 4	503	3.3×10^{-49}	Y	Y	Y	Y	Y	N	62
At4g29350	PFN2, profilin 2	426	4.7×10^{-41}	Y	Y	N	N	N	Y	62
At4g31700	RPS6, 40S ribosomal protein S6	590	2.0×10^{-58}	Y	Y	N	N	N	Y	63
At4g33070	Pyruvate decarboxylase, putative	1790	5.7×10^{-257}	N	Y	N	N	N	Y	25
At4g35830	Aconitate hydratase, cytoplasmic	4087	0.	Y	Y	Y	N	N	N	45
At4g38510	Vacuolar ATP synthase subunit B4	2354	2.3×10^{-245}	Y	Y	Y	N	N	N	6
At4g39800	Inositol-3-phosphate synthase 1	2380	4.1×10^{-248}	N	Y	N	N	N	Y	89
At5g01410	ATPDX1.3, pyridoxine biosynthesis protein 1.3	1377	7.9×10^{-142}	N	Y	Y	N	N	Y	78
At5g01600	ATFER1, ferritin 1	767	3.5×10^{-77}	Y	Y	Y	N	N	N	15
At5g02490	HSC70−2, heat shock cognate 70 kDa protein 2	2321	7.3×10^{-242}	Y	Y	N	N	Y	N	1, 47, 51
At5g02500	HSC70−1, heat shock cognate 70 kDa protein 1	2340	7.1×10^{-244}	Y	Y	N	Y	N	N	1, 47, 51
At5g03290	Isocitrate dehydrogenase, putative	1504	2.8×10^{-155}	Y	Y	N	N	N	Y	36
At5g03300	ADK 2, adenosine kinase 2	1528	7.9×10^{-158}	Y	Y	N	N	N	Y	32
At5g03520	ATRAB8C, ras-related protein RAB8C	801	8.6×10^{-81}	Y	Y	N	N	N	Y	67
At5g09550	ATGDI1, RAB GDP dissociation inhibitor 1	1964	5.0×10^{-204}	N	Y	N	Y	N	N	70
At5g10170	Inositol-3-phosphate synthase 3	2398	5.1×10^{-250}	N	Y	N	N	N	Y	89
At5g10360	RPS6B, 40S ribosomal protein S6−2	576	6.0×10^{-57}	Y	Y	N	N	N	Y	63
At5g15490	UDP-glucose 6-dehydrogenase, putative	2326	2.2×10^{-242}	Y	Y	Y	N	N	N	8
At5g17310	UGP, UDP-glucose pyrophosphorylase	1675	2.1×10^{-173}	N	Y	Y	N	N	N	81
At5g23900	60S ribosomal protein L13C	660	7.6×10^{-66}	Y	Y	N	N	N	Y	34
At5g28540	BIP1, luminal binding protein	2807	2.3×10^{-293}	Y	Y	N	Y	Y	N	5, 11, 48
At5g35360	CAC2, biotin carboxylase subunit	2245	8.3×10^{-234}	Y	Y	N	N	N	Y	21
At5g35590	PAA1, 20S proteasome alpha subunit A1	1128	1.9×10^{-115}	Y	Y	N	N	N	Y	26
At5g42020	BIP2, luminal binding protein	2816	2.6×10^{-294}	Y	Y	N	Y	Y	N	5, 11, 48
At5g42080	ADL1A, *Arabidopsis* dynamin-like protein 1A, GTPase	2675	2.3×10^{-279}	Y	Y	N	N	N	Y	53
At5g43330	Malate dehydrogenase, cytosolic	1525	1.6×10^{-157}	N	Y	Y	Y	N	N	59
At5g43940	ADH2, alcohol dehydrogenase 2	1146	2.4×10^{-117}	Y	Y	N	N	N	Y	23
At5g45775	60S ribosomal protein L11D	830	7.3×10^{-84}	Y	Y	N	Y	N	N	61
At5g48230	ACAT2, cytosolic acetoacetyl-coenzyme A thiolase 2	1565	9.5×10^{-162}	N	Y	N	N	N	Y	94
At5g52640	ATHSP90.1, heat shock potein 90	1709	1.9×10^{-278}	Y	Y	N	N	N	Y	10
At5g55190	RAN3, GTP/ binding protein	1082	1.4×10^{-110}	N	Y	N	N	N	Y	90
At5g56010	HSP81−3, heat shock protein 81−3	1785	5.2×10^{-188}	N	Y	N	N	N	Y	56
At5g56030	HSP81−2, heat shock protein 81−2	1787	3.2×10^{-188}	N	Y	N	N	N	Y	56

Table 2. Continued

AGI name	protein	score	*E*-value	EPP excl.	EPP incl.	pollen proteome			pollen transcriptome	ID tobacco
						Noir	Holmes	Sheoran		
At5g59290	UXS3, UDP-xylose synthase 3	1201	1.7×10^{-126}	N	Y	N	N	N	Y	91
At5g59370	ACT4, actin 4	1854	2.3×10^{-192}	Y	Y	Y	N	Y	N	4, 9, 28, 30, 33
At5g59840	Ras-related GTP-binding family protein	856	1.3×10^{-86}	Y	Y	N	N	N	Y	67
At5g60390	eEF-1-alpha	2277	3.4×10^{-237}	Y	Y	N	Y	N	N	7, 50

[a] *Arabidopsis* orthologues were identified by BLAST; appropriate "score" and "*E*-value" data are shown. For each gene, presence to EPPs as identified by exclusive ("EPP excl.") and inclusive ("EPP incl.") approach is marked (Y = yes; N = no; for detailed description see text). Presence of identified genes in available *Arabidopsis* mature pollen proteomic data sets is shown, "Noir", Noir et al.;[44] "Holmes", Holmes-Davis et al.;[43] "Sheoran", Sheoran et al.[45] Similarly, presence of identified genes within *A. thaliana* male gametophyte data set is indicated, "pollen transcriptome", Honys et al.[42] IDs of original tobacco proteins are given in the "ID tobacco" column.

complexes during pollen tube growth together with gradual decrease of quantity of EPP proteins favors the combined hypothesis of massive onset of translation of transcripts stored in EPPs followed by a continuous activation of others throughout at least the first 24 h of the progamic phase. A number of transcripts were previously shown to be associated with EPPs[33] accompanying previously identified *ntp303* mRNA. Several of these were identified in this study (Figure 3). This demonstrated the common occurrence of these complexes and their broader involvement in mRNA storage. Considering a four day-long tobacco pollen tube growth facilitated by intensive translation but inactive transcription, such essential and abundant store of transcripts for progamic phase would be expected. Moreover, the presence of EPPs in 24 h-old pollen tubes when the first callose plug had been already formed proved trafficking of EPP complexes together with polysomal structures to the pollen tube tip. Although pollen rehydration may represent the first impulse triggering EPP activation, it is unlikely to be acting in later phases; another regulatory mechanism(s) shall be employed. The apparent stability of EPPs and a variety of associated transcripts changed our view of these complexes and presented them as a vital source of information for the progamic phase of male gametophyte development.

The first demand for further analyses of EPP complexes was the estimation of EPP's size. However, although standardized and optimized for higher yield, size exclusion chromatography did not clearly answer this question. Nevertheless, the observed size of EPPs over 8 MDa was higher than that of whole ribosomes (4.2 MDa).[49] Scanning electron microscopy displayed the EPP pellet as a heavy multiprotein complex of heterogeneous components ranging from 20 to 60 nm. According to their size, they were considered to contain ribosomal subunits. If proven, this hypothesis would be in accord with rapid activation and long-term translatability of EPP-associated mRNAs as suggested above. Subsequently, the presence of large and small ribosomal subunits in EPP complexes was demonstrated by two lines of evidence, RT-PCR and mass spectrometry. All these findings made the supramolecular character of EPP complexes obvious and confirmed the original concept of EPP complexes as storage and transport machinery for gradually activated translationally silent mRNAs.

Moreover, the revolutionary insight into the whole concept of RNA storage and translation was achieved by proteomic analyses of EPP complexes purified from mature pollen and *in vitro*-cultivated 4-h pollen tubes. Functional classification of EPP-associated proteins (Figure 6) revealed several well-characterized and distinct clusters. Their composition not only confirmed previously assigned function of EPP complexes, but also enabled the association of new functions in RNA and protein metabolism and transport.

The first large cluster contained proteins directly involved in protein synthesis. Besides ribosomal proteins of both small (3 proteins) and large (9 proteins) ribosomal subunits, proteomic analyses confirmed the presence of several accompanying subunits of eukaryotic initiation (eIF3 and eIF4A) and elongation (eEF1 and eEF2) factors as well as poly(A)-binding proteins confirming the association of translatable mRNA. In the second cluster formed by cytoskeletal proteins, the actin-based system was more abundant with six actins and associated profilin. On the contrary, tubulin cytoskeleton was represented by single tubulin α. These findings prove fluorescence microscopy observations where stronger signal was associated with actin than tubulin (Supplementary Figure 2 in Supporting Information). The third cluster was formed by 26S proteasomal subunits proteins. More proteasomal proteins were observed during the progamic phase (4-h pollen tubes) than in mature pollen. 26S proteasomes were repeatedly detected within ribosomal and/or polysomal fraction due to the similar molecular weight and nonspecific functional interaction between proteasomes and ribosomes as they are ready for proteolysis of nascent proteins.[50] The importance of the selective proteolytic activity associated with 26S proteasome system during pollen germination and tube growth was repeatedly demonstrated.[51−53] Moreover, a number of transcripts encoding proteasome-associated and F-box proteins were identified in *Arabidopsis* developing and germinating pollen.[42,54] Proteasomes were also demonstrated to perform other nonproteolytic functions including transcriptional regulation,[55−57] RNase activity,[58,59] translational regulation,[60,61] and possessing cytoskeleton-binding properties.[52,62] With regard to all above-mentioned alternative functions, the presence of proteasome subunits within EPP complexes is natural in relation to complex EPP-associated spatially and temporarily regulated activities.

Ribosomal and cytoskeletal proteins were accompanied by distinct groups of proteins involved in protein transport, translational and post-translational modifications including HSP chaperones, luminal proteins, protein kinases, and phosphatases. The presence of all these proteins suggested involvement of EPPs also in at least some steps of protein processing and targeting. However, proteomic analyses also revealed other less expected proteins and/or protein complexes associated with EPP complexes. These comprised ER luminal proteins, Golgi network-associated proteins involved in the synthesis of cell wall saccharide precursors, mitochondrial enzymes of energy metabolism, enzymes of general metabolism, and histones.

EPPs, besides their functional association with cytoskeleton, can be organized also in proximity to membrane compartments like ER and Golgi network. Such close structural organization together with protein translocation to ER and Golgi system

explains the presence of ER membrane-associated and luminal proteins together with Golgi-associated proteins and cell wall precursors within EPP complexes.

With respect to mitochondria, their close proximity to EPP complexes is likely since RNA storage, transport, and translation are high energy-demanding processes. Moreover, immature mitochondria of pollen grains undergo soon after the onset of progamic phase (up to 30 min of pollen activation) rapid development[63] involving massive synthesis and turnover of nuclear-encoded mitochondrial proteins (Čapková et al., unpublished data). Therefore, these specific metabolic processes and implied spatial proximity enlighten the appearance of specific mitochondrial metabolic enzymes (Krebs cycle enzymes) as well as enzymes involved in cytosolic glycolysis within the EPP fraction. The latter even can be spatially organized close to EPPs. Finally, histones are extremely unlikely to play any role in RNA cytoplasmic metabolism. However, they can be processed and transported within EPPs.

High-order RNP complexes were repeatedly found to be associated with the cytoskeleton.[23,25,64] Various proteins bound to *cis*-elements in mRNAs were identified to facilitate this association. Cytoskeleton and cytoskeleton-binding proteins arrange special cytoarchitecture and they also contribute to precise functional compartmentalization.[65−67] Cytoskeleton with associated components of translational machinery and translationally silent mRNP molecules can form one of these specific compartments poised for mRNA trafficking, storage, and, once activated, also for localized translation.[50,68] Microtubules have been already demonstrated to serve as high-throughput track for mRNP trafficking,[5] while actin seems to be connected with anchoring and translational activation of mRNA. However, its role in mRNP translocation has been described too.[23,25]The presence of mRNAs encoding cytoskeletal and cytoskeleton-binding proteins in EPP complexes is therefore not surprising since their localization is likely to be under precise spatial and temporal control. The targeting of EPPs to the specific cellular regions and localized translation is analogous to similar processes in neurons, oligodendrocytes, fibroblasts and other more intensively studied systems. For instance, *β-actin* mRNA binds to zipcode-binding protein 1 (ZBP1) that functions as a translational regulator and helps the targeting of the silent mRNA to the synapses.[26,69] Similarly, *EF1*α mRNA is localized to dendrites, and mRNAs encoding *β*-actin, α-tubulin, *κ*-opioid receptor, ADF, RhoA, and cofilin were detected in growing neural axons.[24,26,69]

In addition, the finding of *eIF4E* and *eIFiso4E* mRNA within EPP complexes strongly supports the idea of translational regulation. Now we can only speculate whether translation and further roles of these translation initiation factors would be involved in later regulation of their co-stored mRNA partners. Concerning the *NTP303* transcript, the final destination and exact function of encoded NTP303, an abundant 69-kDa pollen tube wall glycoprotein,[29] remains unclear. Nevertheless, NTP303 was reported to be closely related to the SKS family.[70] Two well-characterized members of this family, SKU5 and SKU6, are localized to the plasma membrane and/or cell wall, playing an important role in the regulation of root directional tip growth and to microtubules with putative function as an intermolecular linker, respectively. Not surprisingly, localization and function of both proteins fit well with the destination of NTP303 close to/in the cell wall and its vital involvement in the pollen tube growth. Moreover, NTP101, the close homologue to NTP303,[30] was identified as being associated with EPPs (this study). The specific function of individual cytoskeleton-associated and/or RNA-binding proteins in these processes can only be speculated, although a number of ribosomal and cytoskeletal proteins also belonged among the highest-ranked proteins. Presence of all identified proteins together with great extent of vigorous post-translational modifications of plant ribosomal proteins[71] suggests high regulatory dynamics within EPP complexes, especially during the progamic phase.

mRNP particles arranged in higher-order complexes or granules were previously identified in many yeast and animal systems. According to their structure, mRNP complexes can be divided into three groups.[23] First, granules composed of many copies of mRNAs and RNA-binding proteins packed together that were identified in *Drosophila* embryos.[72−74] These granules are formed by mRNA−mRNA interactions resulting in dsRNA-stabilized complexes. Second, mRNPs arranged around membrane structures of rough ER were described in *Xenopus* oocytes.[75] Third, large RNP granules containing mRN A, mRNA-binding proteins, and translational apparatus components including several hundreds of ribosomes were repeatedly found in intensively studied neurons and oligodendrites.[25,69,76] Our results fit EPP complexes well with the last model of complex RNP granules containing repressed mRNA in association with preformed translational apparatus. Moreover, both structures—neurons and pollen tubes—share directional growth highlighting the necessity of directional transport of stored mRNA and translational apparatus.

5. Conclusions

Collectively, our results provide the first structural and proteomic data on large ribonucleoprotein complexes in tobacco male gametophyte co-sedimenting with polysomes, EPPs. EPP complexes formed during pollen maturation[33] were found to be present in growing pollen tubes for over 24 h. Although massively activated at early phases of pollen germination, they also serve as a long-term storage of mRNA, transported along with the translational machinery to the tip region. Moreover, EPP complexes were shown to contain ribosomal subunits, rRNAs, and a set of mRNAs. Such preformation and temporal inactivation of the whole translational apparatus fits well with the demand of rapid activation of various reserves at the onset of the progamic phase. To our best knowledge, it is also the first report of stored mRNP complexes containing preformed ribosomes in plants. Presented results shift our view of EPP complexes from the mere RNA storage compartment, in particular stages of pollen development to the complex and well-organized machinery devoted to mRNA storage, transport, and subsequent controlled activation resulting in protein synthesis, processing, selective degradation, and precise localization.

Such an organization is extremely useful in a fast tip-growing structure like a pollen tube. There, massive and orchestrated protein synthesis, processing, and transport must take place in accurately localized regions. Moreover, the presented complex role of EPPs in tobacco cytoplasmic mRNA and protein metabolism makes them likely to be active in another plant species too. The expression of a vast majority of the closest orthologues of EPP proteins also in *Arabidopsis* male gametophyte further extended this concept from tobacco to *Arabidopsis* representing model species with advanced tricellular pollen. Thus, if proven, the utilization of EPP machinery for the localization of complex cytoplasmic gene expression events

can represent a common phenomenon during the progamic phase of male gametophyte development and function.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EPP, EDTA/puromycin-resistant RNP particle co-sedimenting with polysomes; MES, 2-(*N*-morpholino)ethanesulfonic acid; mRNP, messenger ribonucleoprotein particle; RNP particle, ribonucleoprotein particle; RT-PCR, reverse transcription−polymerase chain reaction; SMM-MES, sucrose mineral media supplemented with MES.

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Supporting Information Available: Supplementary Figure 1, electron micrographs of EPP fraction isolated from 4 h pollen tubes. The image shows individual particles variable in their size. Scale bar = 100 nm. Supplementary Figure 2, epifluorescent micrographs of the EPP fraction isolated from 4 h pollen tubes. The presence of actin (A, B) and tubulin (C, D) was visualized by rhodamine-phalloidin and FITC-IgG immunodetection, respectively, within EPP (A, C) and polysomal fractions (B, D). Scale bar = 50 μm. Supplementary Table 1, sequence-specific primers used for RT-PCR analyses. Supplementary Table 2, all proteins identified by mass spectrometry analyses in EPPs isolated from mature pollen and 4-h pollen tubes. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Čapková, V.; Hrabětová, E.; Tupý, J. Protein synthesis in pollen tubes: Preferential formation of new species independent of transcription. *Sex. Plant Reprod.* **1988**, *1*, 150–155.
(2) Twell, D. Pollen developmental biology. In *Plant Reproduction. Annual Plant Reviews*; O'Neil, S. D.; Roberts, J. A., Eds.; Sheffield Academic Press: Sheffield, U.K., 2002; Vol. 6, pp 86−153.
(3) Spirin, A. S.; Nemer, M. Messenger RNA in early sea urchin embryos: Cytoplasmic particles. *Science* **1965**, *150*, 214–216.
(4) Kleene, K. C. Patterns, mechanisms, and functions of translation regulation in mammalian spermatogenic cells. *Cytogenet. Genome Res.* **2003**, *103* (3−4), 217–224.
(5) Carson, J. H.; Barbarese, E. Systems analysis of RNA trafficking in neural cells. *Biol. Cell* **2005**, *97* (1), 51–62.
(6) Colegrove-Otero, L. J.; Minshall, N.; Standart, N. RNA-binding proteins in early development. *Crit. Rev. Biochem. Mol. Biol.* **2005**, *40* (1), 21–73.
(7) Du, T. G.; Schmid, M.; Jansen, R. P. Why cells move messages: the biological functions of mRNA localization. *Semin. Cell Dev. Biol.* **2007**, *18* (2), 171–177.
(8) Macdonald, P. Diversity in translational regulation. *Curr. Opin. Cell Biol.* **2001**, *13* (3), 326–331.
(9) Palacios, I. M.; St Johnston, D. Getting the message across: the intracellular localization of mRNAs in higher eukaryotes. *Annu. Rev. Cell Dev. Biol.* **2001**, *17*, 569–614.
(10) Ajtkhozhin, M. A.; Doschanov, K. J.; Akhanov, A. U. Informosomes as a stored form of mRNA in wheat embryos. *FEBS Lett.* **1976**, *66* (1), 124–126.
(11) Krochko, J. E.; Pramanik, S. K.; Bewley, J. D. Contrasting storage protein synthesis and messenger RNA accumulation during development of zygotic and somatic embryos of alfalfa (Medicago sativa L.). *Plant Physiol.* **1992**, *99* (1), 46–53.
(12) Pramanik, S. K.; Krochko, J. E.; Bewley, J. D. Distribution of cytosolic mRNAs between polysomal and ribonucleoprotein complex fractions in alfalfa embryos: stage-specific translational repression of storage protein synthesis during early somatic embryo development. *Plant Physiol.* **1992**, *99* (4), 1590–1596.
(13) Choi, S. B.; Wang, C.; Muench, D. G.; Ozawa, K.; Franceschi, V. R.; Wu, Y.; Okita, T. W. Messenger RNA targeting of rice seed storage proteins to specific ER subdomains. *Nature* **2000**, *407* (6805), 765–767.
(14) Li, X.; Franceschi, V. R.; Okita, T. W. Segregation of storage protein mRNAs on the rough endoplasmic reticulum membranes of rice endosperm cells. *Cell* **1993**, *72*, 869–879.
(15) Apuya, N. R.; Zimmerman, J. L. Heat shock gene expression is controlled primarily at the translational level in carrot cells and somatic embryos. *Plant Cell* **1992**, *4*, 657–665.
(16) Stuger, R.; Ranostaj, S.; Materna, T.; Forreiter, C. Messenger RNA-binding properties of nonpolysomal ribonucleoproteins from heat-stressed tomato cells. *Plant Physiol.* **1999**, *120* (1), 23–31.
(17) Wood, A. J.; Oliver, M. J. Translational control in plant stress: the formation of messenger ribonucleoprotein particles (mRNPs) in response to desiccation of Tortula ruralis gametophytes [Full text delivery]. *Plant J.* **1999**, *18* (4), 359–370.
(18) Abe, S.; Ito, Y.; Davies, E. Association of cytoskeletal proteins in the membrane-bound polysome fraction from peas using conventional polysome isolation buffers. *Plant Physiol. Biochem.* **1994**, *32* (4), 547–554.
(19) Abe, S.; Ito, Y.; Davies, E. Isolation of a heparin sensitive, ribosome sedimenting factor from the cytoskeleton fractions of peas and corn. *Plant Physiol. Biochem.* **1995**, *33* (4), 463–470.
(20) Davies, E.; Abe, S.; Larkins, B. A.; Clare, A. M.; Quartano, R. S.; Weidner, S., The role of the cytoskeleton in plant protein synthesis. In *A Look beyond Transcription: Mechanisms Determining mRNA Stability and Translation in Plants*; Bailey-Serres, J., Gallie, D. R., Eds.; American Society of Plant Physiologists: Rockwell, MD, 1998; pp 115−123.
(21) Davies, E.; Fillingham, B. D.; Abe, S. The plant cytoskeleton. In *The Cytoskeleton*; Hesketh, J. E.; Pryme, I. F., Eds.; JAI Press: New York, 1996; pp 405−449.
(22) Ovchinnikov, L. P.; Skabkin, M. A.; Ruzanov, P. V.; Evdokimova, V. M. Major mRNP proteins in the structural organization and function of mRNA in eukaryotic cells. *Mol. Biol.* **2001**, *35* (4), 548–558.
(23) Jansen, R. P. mRNA localization: message on the move. *Nat. Rev. Mol. Cell Biol.* **2001**, *2* (4), 247–256.
(24) Hengst, U.; Jaffrey, S. R. Function and translational regulation of mRNA in developing axons. *Semin. Cell Dev. Biol.* **2007**, *18* (2), 209–215.
(25) Bassell, G.; Singer, R. H. mRNA and cytoskeletal filaments. *Curr. Opin. Cell Biol.* **1997**, *9* (1), 109–115.
(26) Dahm, R.; Kiebler, M.; Macchi, P. RNA localisation in the nervous system. *Semin. Cell Dev. Biol.* **2007**, *18* (2), 216–223.
(27) Han, J. R.; Yiu, G. K.; Hecht, N. B. Testis/brain RNA-binding protein attaches translationally repressed and transported mRNAs to microtubules. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 9550–9554.
(28) Jansen, R.-P. RNA-cytoskeletal associations. *FASEB J.* **1999**, *13*, 455–466.
(29) Weterings, K.; Reijnen, W.; van Aarssen, R.; Kortstee, A.; Spijkers, J.; van Herpen, M.; Schrauwen, J.; Wullems, G. Characterization of a pollen-specific cDNA clone from Nicotiana tabacum expressed during microgametogenesis and germination. *Plant Mol. Biol.* **1992**, *18* (6), 1101–1111.
(30) Wittink, F. R. A.; Knuiman, B.; Derksen, J.; Capkova, V.; Twell, D.; Schrauwen, J. A. M.; Wullems, G. J. The pollen-specific gene Ntp303 encodes a 69 kD glycoprotein associated with the vegetative membranes and the cell wall. *Sex. Plant Reprod.* **2000**, *12*, 276–284.
(31) Čapková, V.; Hrabětová, E.; Tupý, J. Protein changes in tobacco pollen culture: A newly synthesized protein related to pollen tube growth. *J. Plant Physiol.* **1987**, *130*, 307–314.
(32) Štorchová, H.; Čapková, V.; Tupý, J. A *Nicotiana tabacum* mRNA encoding a 69-kDa glycoprotein occuring abundantly in pollen tubes is transcribed but not translated during pollen development in the anthers. *Planta* **1994**, *192*, 441–445.
(33) Honys, D.; Combe, J. P.; Twell, D.; Čapková, V. The translationally repressed pollen-specific ntp303 mRNA is stored in non-polysomal mRNPs during pollen maturation. *Sex. Plant Reprod.* **2000**, *13*, 135–144.
(34) Petru, E.; Hrabetova, E.; Tupy, J. The technique of obtaining germinating pollen without microbial contamination. *Biol. Planta.* **1964**, *6* (1), 68–69.
(35) Tupý, J.; Říhová, L. Changes and growth effect of pH in pollen tube culture. *J. Plant Physiol.* **1984**, *115*, 1–10.
(36) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227* (5259), 680–5.
(37) Schaffner, W.; Weissmann, C. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Anal. Biochem.* **1973**, *56* (2), 502–514.
(38) Sammons, D. W.; Adams, L. D.; Nishizawa, E. E. Ultrasensitive silver-based color staining of polypeptides in polyacrylamide gels. *Electrophoresis* **1981**, *2*, 135–141.

(39) Mittermann, I.; Swoboda, I.; Pierson, E.; Eller, N.; Kraft, D.; Valenta, R.; Heberle-Bors, E. Molecular cloning and characterization of profilin from tobacco (Nicotiana tabacum): increased profilin expression during pollen maturation. *Plant Mol. Biol.* **1995**, *27*, 137–146.
(40) Chen, C. Y.; Wong, E. I.; Vidali, L.; Estavillo, A.; Hepler, P. K.; Wu, H. M.; Cheung, A. Y. The regulation of actin organization by actin-depolymerizing factor in elongating pollen tubes. *Plant Cell* **2002**, *14* (9), 2175–2190.
(41) Combe, J. P.; Petracek, M. E.; van Eldik, G.; Meulewaeter, F.; Twell, D. Translation initiation factors eIF4E and eIFiso4E are required for polysome formation and regulate plant growth in tobacco. *Plant Mol. Biol.* **2005**, *57* (5), 749–760.
(42) Honys, D.; Twell, D. Transcriptome analysis of haploid male gametophyte development in Arabidopsis. *GenomeBiology* **2004**, *5* (11), R85.
(43) Holmes-Davis, R.; Tanaka, C. K.; Vensel, W. H.; Hurkman, W. J.; McCormick, S. Proteome mapping of mature pollen of Arabidopsis thaliana. *Proteomics* **2005**, *5* (18), 4864–4884.
(44) Noir, S.; Brautigam, A.; Colby, T.; Schmidt, J.; Panstruga, R. A reference map of the Arabidopsis thaliana mature pollen proteome. *Biochem. Biophys. Res. Commun.* **2005**, *337* (4), 1257–1266.
(45) Sheoran, I. S.; Sproule, K. A.; Olson, D. J. H.; Ross, A. R. S.; Sawhney, V. K. Proteome profile and functional classification of proteins in Arabidopsis thaliana (Landsberg erecta) mature pollen. *Sex. Plant Reprod.* **2006**, *19* (4), 185–196.
(46) Pina, C.; Pinto, F.; Feijo, J. A.; Becker, J. D. Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. *Plant Physiol.* **2005**, *138* (2), 744–756.
(47) Zimmermann, P.; Hennig, L.; Gruissem, W. Gene-expression analysis and network discovery using Genevestigator. *Trends Plant Sci.* **2005**, *10* (9), 407–409.
(48) Čapková, V.; Hrabětová, E.; Tupý, J.; Říhová, L. Amino acid uptake and protein synthesis in cultured tobacco pollen. *Biochem. Physiol. Pflanzen* **1983**, *178*, 511–520.
(49) Alberts, B.; Bray, D.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walters, P. *Essential Cell Biology*; Garland Publishing, Inc.: New York, 1998.
(50) Chuong, S. D.; Park, N. I.; Freeman, M. C.; Mullen, R. T.; Muench, D. G. The peroxisomal multifunctional protein interacts with cortical microtubules in plant cells. *BMC Cell Biol.* **2005**, *6*, 40.
(51) Speranza, A.; Scoccianti, V.; Crinelli, R.; Calzoni, G. L.; Magnani, M. Inhibition of proteasome activity strongly affects kiwifruit pollen germination. Involvement of the ubiquitin/proteasome pathway as a major regulator. *Plant Physiol.* **2001**, *126* (3), 1150–1161.
(52) Sheng, X.; Hu, Z.; Lu, H.; Wang, X.; Baluska, F.; Samaj, J.; Lin, J. Roles of the ubiquitin/proteasome pathway in pollen tube growth with emphasis on MG132-induced alterations in ultrastructure, cytoskeleton, and cell wall components. *Plant Physiol.* **2006**, *141* (4), 1578–1590.
(53) Speranza, A.; Ferri, P.; Battistelli, M.; Falcieri, E.; Crinelli, R.; Scoccianti, V. Both trivalent and hexavalent chromium strongly alter in vitro germination and ultrastructure of kiwifruit pollen. *Chemosphere* **2007**, *66* (7), 1165–1174.
(54) Wang, Y.; Zhang, W. Z.; Song, L. F.; Zou, J. J.; Su, Z.; Wu, W. H. Transcriptome analyses show changes in gene expression to accompany pollen germination and tube growth in Arabidopsis. *Plant Physiol.* **2008**, *148* (3), 1201–1211.
(55) Baker, S. P.; Grant, P. A. The proteasome: not just degrading anymore. *Cell* **2005**, *123* (3), 361–3.
(56) Gillette, T. G.; Gonzalez, F.; Delahodde, A.; Johnston, S. A.; Kodadek, T. Physical and functional association of RNA polymerase II and the proteasome. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (16), 5904–5909.
(57) Lee, D.; Ezhkova, E.; Li, B.; Pattenden, S. G.; Tansey, W. P.; Workman, J. L. The proteasome regulatory particle alters the SAGA coactivator to enhance its interactions with transcriptional activators. *Cell* **2005**, *123* (3), 423–436.
(58) Jarrousse, A. S.; Petit, F.; Kreutzer-Schmid, C.; Gaedigk, R.; Schmid, H. P. Possible involvement of proteasomes (prosomes) in AUUUA-mediated mRNA decay. *J. Biol. Chem.* **1999**, *274* (9), 5925–5930.
(59) Petit, F.; Jarrousse, A. S.; Boissonnet, G.; Dadet, M. H.; Buri, J.; Briand, Y.; Schmid, H. P. Proteasome (prosome) associated endonuclease activity. *Mol. Biol. Rep.* **1997**, *24* (1−2), 113–117.
(60) Huang, X.; Hetfeld, B. K.; Seifert, U.; Kahne, T.; Kloetzel, P. M.; Naumann, M.; Bech-Otschir, D.; Dubiel, W. Consequences of COP9 signalosome and 26S proteasome interaction. *FEBS J.* **2005**, *272* (15), 3909–3917.
(61) Yahalom, A.; Kim, T. H.; Roy, B.; Singer, R.; von Arnim, A. G.; Chamovitz, D. A. Arabidopsis eIF3e is regulated by the COP9 signalosome and has an impact on development and protein translation. *Plant J* **2008**, *53* (2), 300–311.
(62) Berger, S.; Liddle, L. B.; Dillard, W. L.; Wittke, W.; Traub, P. Cytoskeletal 10 nm filaments in cells of the algal phyla Chlorophyta, Charophyta, and Chrysophyta and their developmentally regulated and species-specific association with prosomes. *Protoplasma* **2003**, *221* (3−4), 277–288.
(63) Hoekstra, F. A.; Bruinsma, J. Respiration and vitality of binucleate and trinucleate pollen. *Physiol. Plant* **1975**, *34*, 221–225.
(64) Antic, D.; Keene, J. D. Messenger ribonucleoprotein complexes containing human ELAV proteins: interactions with cytoskeleton and translational apparatus. *J. Cell Sci.* **1998**, *111* (Pt. 2), 183–197.
(65) Giorgi, C.; Moore, M. J. The nuclear nurture and cytoplasmic nature of localized mRNPs. *Semin. Cell Dev. Biol.* **2007**, *18* (2), 186–193.
(66) Luby-Phelps, K. Effect of cytoarchitecture on the transport and localization of protein synthetic machinery. *J. Cell Biochem.* **1993**, *52* (2), 140–147.
(67) Luby-Phelps, K.; Weisiger, R. A. Role of cytoarchitecture in cytoplasmic transport. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **1996**, *115B*, 295–306.
(68) Muench, D. G.; Park, N. I. Messages on the move: the role of the cytoskeleton in mRNA localization and translation in plant cells. *Can. J. Bot.* **2006**, *84*, 572–580.
(69) Hirokawa, N. mRNA transport in dendrites: RNA granules, motors, and tracks. *J. Neurosci.* **2006**, *26* (27), 7139–7142.
(70) de Groot, P.; Weterings, K.; de Been, M.; Wittink, F.; Hulzink, R.; Custers, J.; van Herpen, M.; Wullems, G. Silencing of the pollen-specific gene NTP303 and its family members in tobacco affects in vivo pollen tube growth and results in male sterile plants. *Plant Mol. Biol.* **2004**, *55* (5), 715–726.
(71) Carroll, A. J.; Heazlewood, J. L.; Ito, J.; Millar, A. H. Analysis of the Arabidopsis cytosolic ribosome proteome provides detailed insights into its components and their post-translational modification. *Mol. Cell. Proteomics* **2008**, *7* (2), 347–369.
(72) Driever, W.; Nusslein-Volhard, C. A gradient of bicoid protein in Drosophila embryos. *Cell* **1988**, *54* (1), 83–93.
(73) Ephrussi, A.; Dickinson, L. K.; Lehmann, R. Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. *Cell* **1991**, *66* (1), 37–50.
(74) Ferrandon, D.; Koch, I.; Westhof, E.; Nusslein-Volhard, C. RNA-RNA interaction is required for the formation of specific bicoid mRNA 3′ UTR-STAUFEN ribonucleoprotein particles. *EMBO J.* **1997**, *16* (7), 1751–1758.
(75) Schroeder, K. E.; Yost, H. J. Xenopus poly (A) binding protein maternal RNA is localized during oogenesis and associated with large complexes in blastula. *Dev. Genet.* **1996**, *19* (3), 268–276.
(76) Elvira, G.; Wasiak, S.; Blandford, V.; Tong, X. K.; Serrano, A.; Fan, X.; del Rayo Sanchez-Carbente, M.; Servant, F.; Bell, A. W.; Boismenu, D.; Lacaille, J. C.; McPherson, P. S.; DesGroseillers, L.; Sossin, W. S. Characterization of an RNA granule from developing brain. *Mol. Cell. Proteomics* **2006**, *5* (4), 635–651.

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