

Sub-plastidial localization of two different phage-type RNA polymerases in spinach chloroplasts

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ABSTRACT

Plant plastids contain a circular genome of ~150 kb organized into ~35 transcription units. The plastid genome is organized into nucleoids and attached to plastid membranes. This relatively small genome is transcribed by at least two different RNA polymerases, one being of the prokaryotic type and plastid-encoded (PEP), the other one being of the phage-type and nucleus-encoded (NEP). The presumed localization of a second phage-type RNA polymerase in plastids is still questionable. There is strong evidence for a sequential action of NEP and PEP enzymes during plant development attributing a prevailing role of NEP during early plant and plastid development, although NEP is present in mature chloroplasts. In the present paper, we have analysed two different NEP enzymes from spinach with respect to subcellular and intra-plastidial localization in mature chloroplasts with the help of specific antibodies. Results show the presence of the two different NEP enzymes in mature chloroplasts. Both enzymes are entirely membrane bound but, unlike previously thought, this membrane binding is not mediated via DNA. This finding indicates that NEP enzymes are not found as elongating transcription complexes on the template DNA in mature chloroplasts and raises the question of their function in mature chloroplasts.

INTRODUCTION

The plastid genome is transcribed by at least three different RNA polymerases. According to their different coding origins these enzymes are called plastid-encoded plastid RNA polymerase (PEP) and nucleus-encoded plastid RNA polymerase (NEP). PEP is a multimeric enzyme of the prokaryotic type and NEP enzymes are monomeric enzymes of the phage-type (1–3). The number of genes coding for NEP

enzymes differs in different plant species, e.g. only two genes have been reported for monocotyledons (4–6) and for the moss *Physcomitrella patens* (7,8), but three genes exist in dicotyledons like *Arabidopsis thaliana* (9,10) and, correspondingly, six genes have been reported in *Nicotiana tabacum*, a natural allotetraploid plant (11). The localization of the corresponding proteins is not yet clear and the functional significance for this multiplicity of enzymes is not well understood. Results using transit peptide-GFP fusion constructs are controversial. It had been shown for *Arabidopsis* that one of the three phage-type RNA polymerases (AtRPOT;3) is imported into chloroplasts, one (AtRPOT;1) is imported into mitochondria and the third one (AtRPOT;2) is targeted to the two organelles (9,10). The dual targeting of AtRPOT;2 has been recently contradicted by Kabeya and Sato (12) who showed exclusively mitochondrial localization of this enzyme. Again in contradiction to an exclusively mitochondrial localization of AtRPOT;2 are results obtained from characterization of knock-out mutants that show changes only in plastid gene expression but not in mitochondrial gene expression (13).

Also not clear is the nomenclature of these enzymes. In different papers, the mitochondrial enzyme is named RPOT;1 or RPOY or RPOTm. The plastid enzyme is named RPOT;3 or RPOZ or RPOTp, and the dual targeted enzyme is named RPOT;2 or RPOX. In the present paper, to assure easy understanding, we will use the nomenclature RPOTm (for mitochondrial enzyme), RPOTp (for plastid enzyme) and RPOTmp (for dual targeted enzyme; mitochondria and plastid).

On the biochemical level, chloroplast RNA polymerases have been characterized so far in two different ways. Either they have been purified as soluble enzymes from supernatants of osmotically shocked and sheared chloroplasts or as protein–DNA complexes (named TAC for transcriptionally active chromosome) after solubilization from plastid membranes by Triton X-100 treatment [for a review see (14)]. A large portion of this DNA-bound RNA polymerase activity can be released from the protein–DNA complexes by salt treatment; but part of the RNA polymerase activity remains tightly bound to DNA, even after high-salt treatment (15,16).

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Soluble RNA polymerases have been characterized by antibody cross-reactions and by protein sequencing of polypeptides present in highly purified transcriptionally active extracts (17–20). These experiments have shown that PEP as well as NEP enzymes can be recovered in form of soluble enzymes from chloroplast extracts. On the other hand, the membrane bound part of the chloroplast enzymes has been much less characterized with respect to the RNA polymerases that are present. Although some major polypeptides have been enriched in purified TAC fractions, none of these polypeptides has been identified unambiguously as subunit of a RNA polymerase (15,16,21). Actually, only the presence of the α -subunit of PEP has been demonstrated in high-salt TAC complexes by immuno-cross-reaction (22) thus indicating the presence of PEP in membrane bound TAC complexes.

Also, the nature of membrane binding of transcriptionally active protein–DNA complexes is not yet clear. It is generally assumed that RNA polymerases are localized to plastid membrane fractions via anchoring of its DNA templates. DNA-binding proteins like PEND (plastid envelope DNA-binding protein) and MFP1 represent reliable candidates for anchoring plastid DNA to plastid membranes (envelopes or thylakoids, respectively) (23–25).

In order to get more information on NEP enzymes, in the present paper we characterize two NEP enzymes from spinach in more detail (accession nos Y18852 and Y18853). These two enzymes are homologous to the *Arabidopsis* RPOTp and RPOTmp enzymes, i.e. to the plastid located and to the presumably dual targeted NEP enzyme. By using specific peptide antibodies to each of these enzymes we address the questions of whether both NEP enzymes are localized in plastids and whether both (one) of the enzymes are (is) attached to plastid membranes.

MATERIALS AND METHODS

Cloning of the spinach *RPOTp* and *RPOTmp* cDNAs

The two cDNAs have been isolated from a commercially available Lamda ZAP®II Library (Stratagene). In a first step, 5000 phages have been screened using a PCR-amplified DNA fragment. Primers (5'-TCGTGGTTCGGGCA-TATCC-3' and 5'-CACCTTCCTTCCAGTGGC-3') had been designed using the sequence of a gene fragment that had been communicated by A. Weihe. From this experiment, one clone corresponding to a partial sequence of *RPOTmp* was obtained. In a second step, this clone was used to re-screen the library and to obtain full-length clones of *RPOTmp* and *RPOTp*.

Building up the phylogenetic tree

Sequences have been aligned by Multalin (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html) and gaps have been removed manually. Only the most conserved C-terminal 441 amino acids (Supplementary Figure 1S) have been selected for tree construction using the PHYLIP program. The alignment was reformatted in PHYLIP using the CLUSTALW program. Trees based on parsimony as well as on distance were constructed by Protpars and ProtDist programs, respectively, which are available on the Pasteur server (<http://bioweb.pasteur.fr/seqanal/phylogeny/intro-uk.html>).

Distances have been obtained by the Jones–Taylor–Thornton Matrix and the phylogenetic tree was constructed by the neighbour-joining method. Bootstraps have been performed before each analysis. The numbers on the branches correspond to bootstrap confidence levels obtained for 100 replicates. Only values above 50 are indicated.

Antibody preparation and purification

Peptide antibodies have been prepared in rabbits by Eurogentec using their standard protocol. Peptides that have been used for RPOTmp and RPOTp antibody production are indicated in Figure 1B. To prepare antibodies against the α subunit of spinach PEP the following peptides have been used: H₂N-WKCVESRTDSKCL-CONH₂; H₂N-HAEENVNLEDNQHK VC-CONH₂. For purification of specific IgG fractions the corresponding peptides have been coupled to activated immunoaffinity supports (Bio-Rad) and the sera have been purified according to the supplier's protocol.

Protein analysis

Proteins have been isolated and analysed by western immunoblotting as described previously (26). Briefly, protein extracts were prepared according to Hurkman and Tanaka (27). Protein concentrations were determined by using the BioRad DC protein assay and BSA as standard. After separation of equal amounts of protein by SDS–PAGE and subsequent transfer to nitrocellulose membranes (Schleicher & Schuell) proteins were either stained by Ponceau S or analysed for antibody reaction using the ECL western blotting detection system (Amersham Biosciences).

Chloroplast sub-fractionation

All operations were carried out at 0–5°C. Intact chloroplasts were obtained from spinach leaves and purified by isopycnic centrifugation using Percoll gradients according to Douce and Joyard (28). Stroma, thylakoids and the envelope fractions were purified from lysed chloroplasts by sucrose gradient centrifugation as described in Douce *et al.* (29).

Preparation of mitochondria

All steps were carried out at 0–5°C. Intact mitochondria were prepared from spinach leaves by isopycnic centrifugation using self-generating Percoll gradients as described by Neuburger *et al.* (30). Mitochondria accumulate as a white tight band near the bottom of the gradient, whereas thylakoids remain near to the top of the gradient. Mitochondria were then concentrated by centrifugation and finally re-suspended in phosphate buffer (10 mM, pH 7.2) containing Mannitol (0.3 M), EDTA (1 mM) and DTT (1 mM).

Membrane washing and DNase treatment

Aliquots containing purified intact chloroplasts corresponding to 60 μ g of chlorophyll were lysed in hypotonic medium (10 mM MOPS, pH 7.8; 4 mM MgCl₂; 1 mM phenylmethylsulfonyl fluoride) and centrifuged immediately at 35 000 *g* for 30 min. The pellets (which is still contaminated by soluble proteins) were re-suspended in hypotonic medium and treated in a final volume of 600 μ l with different concentrations of DNase by gentle agitation at room temperature for 1 h. The DNase reaction was stopped by addition of 10 mM EDTA

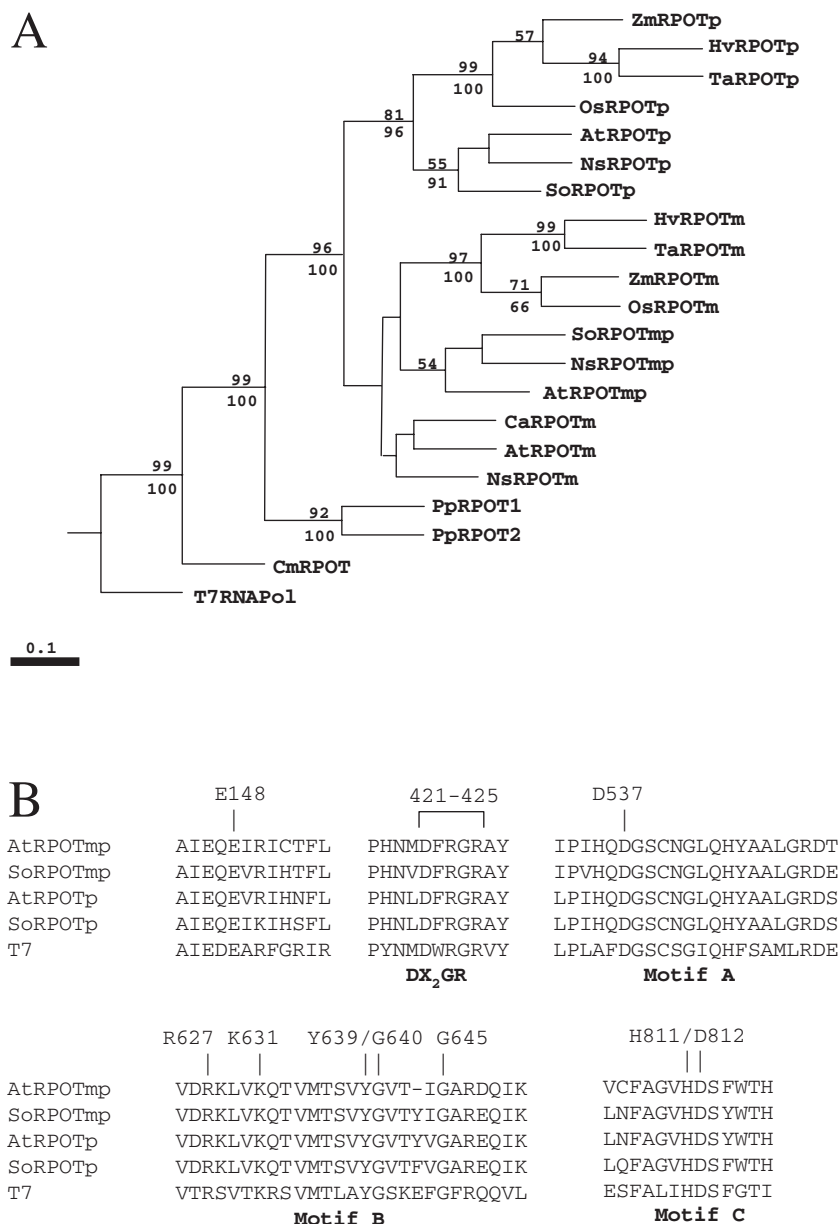


Figure 1. Sequence analyses of the two spinach plastid phage-type RNA polymerases. (A) Phylogenetic analysis of the two spinach enzymes. The alignment that has been used for tree construction is shown in Supplementary Figure 1S. The parsimony tree is shown including bootstrap confidence values obtained from parsimony (upper values) and neighbour-joining (lower values) analyses. (B) Conservation of functionally important amino acids and motifs in the two spinach phage-type RNA polymerases. Numbering on the top of the conserved sequence blocks indicate the position of the corresponding amino acid and annotations below the sequence blocks indicate the name of the corresponding motif in the T7 RNA polymerase sequence. Abbreviations and accession nos are At (*A.thaliana*, Y08463, Y08137, AJ001037), Ca (*Chenopodium album*, Y08067), Hv (*Hordeum vulgare*, HVU507395, AJ586899), Ns (*Nicotiana glauca*, AJ302020, AJ302019, AJ416568), Pp (*P.patens*, AJ416854, AJ416855), So (*Spinacia oleracea*, Y18852, Y18853), Ta (*Triticum aestivum*, U34402, AF091838), T7 (T7 RNA polymerase, AAB28111) and Zm (*Zea mays*, AF127022, AJ005343). The Cm (*C.merolae*) sequence has been obtained from the entry of the *Cyanidioschyzon* project page (CMJ257C) and the sequence has not yet been experimentally verified.

and reaction mixtures were centrifuged at 5000 *g* for 15 min. Proteins of the soluble fractions (S2, 4, 6 and 8) were precipitated by TCA (10% final volume) and directly solubilized in loading buffer for SDS-PAGE. Proteins of the pellets (M1, 3, 5 and 7) were solubilized in 20 μ l of hypotonic medium. Aliquots corresponding to 7.5 μ g of chlorophyll have been used for western immunoblotting. Chloroplast DNA degradation was controlled by analysing aliquots of the DNase treated

membrane fractions (equivalent to 4 μ g of chlorophyll) by gel electrophoretic separation on 0.8% agarose gels and ethidium bromide staining of DNA.

Thermolysin treatment

Thermolysin treatment was performed essentially as described by Joyard *et al.* (31). Briefly, the chlorophyll concentration of

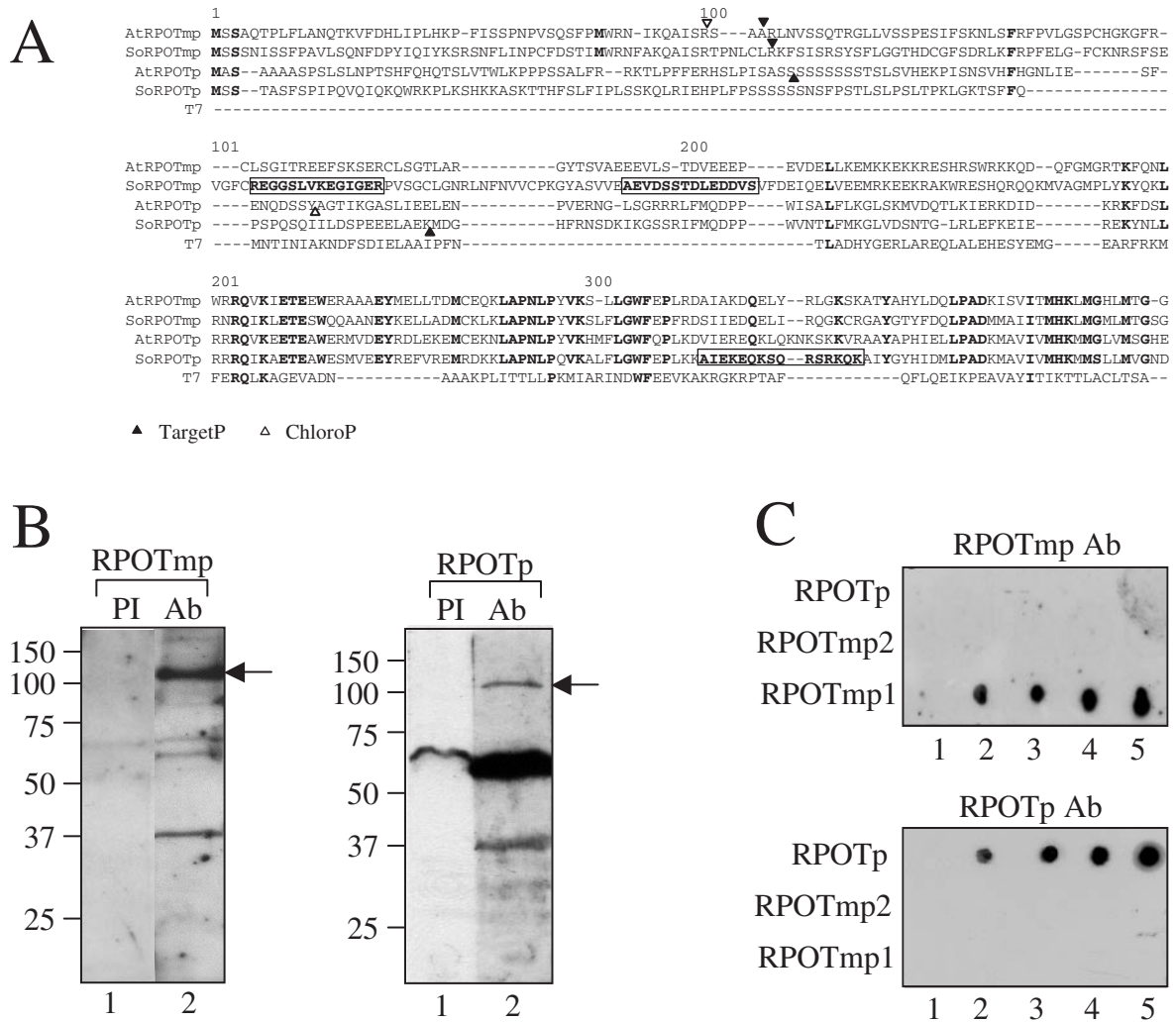


Figure 2. Characterization of RPOTp and RPOTmp specific antibodies. (A) Alignment of the N-terminal parts of the *Arabidopsis* and spinach RPOTmp and RPOTp amino acid sequences. Predicted target sequence cleavage sites are indicated by closed (TargetP) or open (ChloroP) triangles. In the case of SoRPOTmp and SoRPOTp, Target P and ChloroP indicate the same cleavage sites. Peptides that have been used for production of specific antibodies are boxed. (B) The antisera (lane 2) and the pre-immune sera (lane 1) of the RPOTmp (left panel) and RPOTp enzymes (right panel) have been analysed by immunoblotting using 40 µg of total spinach protein extracts. (C) The specificity of the RPOTmp and RPOTp antibodies has been verified by dot blot analyses on all three peptides. 5, 50, 100, 150 and 200 ng (lanes 1–5, respectively) of peptides (indicated as RPOTp, RPOTmp2 and RPOTmp1 on the left panel of the figure) have been spotted onto nitrocellulose filter and the antibody cross-reactions have been tested using purified antibodies, i.e. peptide specific IgG fractions of the RPOTmp (left) and RPOTp (right) antibodies.

isolated intact chloroplast was adjusted to 1 mg/ml. Plastids were incubated for 1 h at 4°C in buffer A [MOPS-NaOH, 10 mM, pH 7.8 containing 0.33 M of Sorbitol, 1 mM of CaCl₂ and different concentrations of thermolysin (Sigma)]. As control, EGTA had been added from the beginning to one of the reactions (10 mM final). All other reactions were stopped after 1 h by addition of EGTA (10 mM). All reactions are loaded on a Percoll cushion (40% in buffer A) and centrifuged at 5000 g (JS 7.5 rotor; Beckman) for 25 min in order to eliminate broken chloroplasts and thermolysin. The pellet of intact chloroplasts was re-suspended in buffer A and remaining Percoll was eliminated by dilution in 10 vol of buffer A and subsequent centrifugation (10 min at 3000 g). The pellet of intact chloroplasts was finally re-suspended in buffer A and sub-plastid fractions were obtained as described above.

RESULTS

Bioinformatic analysis of two different phage-type RNA polymerases from spinach

To get a first idea to which type of the enzymes, RPOTm, RPOTmp or RPOTp, the two spinach NEP sequences belong, we performed a phylogenetic analysis using hitherto known NEP sequences including monocotyledonous plants and the red alga *Cyanidioschyzon merolae*. Parsimony and neighbour-joining analyses have been used to construct the corresponding trees. Figure 1A shows the parsimony tree with bootstrap confidence values for branching above 50 out of 100 replicates using the two methods.

The tree shows distinct clades grouping plastid RPOTp(s) away from mitochondrial RPOTm(s) and RPOTmp(s). Within

the RPOTm/mp group only the monocotyledonous RPOTm enzymes form a solid clade. The two spinach phage-type enzymes analysed here group with RPOTp and RPOTm/RPOTmp, respectively. Although SoRPOTmp groups together with other RPOTmp(s) bootstrap values are not solid and do not permit clear separation of RPOTm(s) and RPOTmp(s).

The two *P.patens* RPOT sequences are clearly separated from *C.merolae* but are also not directly related to the plastid and mitochondrial groups. The two *P.patens* genes resulted probably from a duplication of an ancestral gene. The two proteins are located in mitochondria as shown recently using specific antibodies (12). Altogether, the phylogenetic data suggest that the plastid NEP appeared after the emergence of terrestrial plants as presented here by the bryophyte *P.patens*.

The predicted size of the two spinach phage-type RNA polymerases RPOTp (Y18853) and RPOTmp (Y18852) is about 101 and 111 kDa, respectively. Sequence alignment with T7 RNA polymerase shows that amino acids that have been shown previously by analysis of mutated T7 RNA polymerase to be important for RNA polymerase activity [D537, R627, Y639, G640, H811, D812; (32)], stabilization of the RNA:DNA hybrid during early stages of transcription initiation [motif DX2GR, residues 421–425; (33)], RNA binding [E148; (34)] and discrimination between NTPs and dNTPs [Y639; (35)] have been conserved (Figure 1B). This indicates that both spinach phage-type enzymes represent a priori transcriptionally active RNA polymerases.

Characterization of RPOTp and RPOTmp specific antibodies

Figure 2A shows the N-terminal part of the protein sequences of the *Arabidopsis* and spinach RPOTp and RPOTmp enzymes. In order to avoid immunological cross-reaction between the two enzymes immunogenic peptides have been designed in the least conserved parts of the protein sequences. The three peptides that are marked by open boxes have been chosen for antibody production. Two-peptides have been used to prepare antibodies against RPOTmp (Double X Program; Eurogentec) and one-peptide has been used to prepare antibodies against RPOTp. Both antibodies reveal polypeptides of about 110 kDa, i.e. of the expected size, but also some additional proteins of lower size are detected (Figure 2B). To reduce the unspecific reactions the antibodies have been purified on the corresponding peptides (Materials and Methods). After purification, the unspecific reactions are much reduced (data not shown).

To exclude cross-reaction between RPOTp and RPOTmp antibodies completely, both antibodies have been tested on different quantities of all three peptides that have been used for antibody production (Figure 2C). This experiment shows that only one of the two RPOTmp peptides has been immunoreactive, but both antibodies, anti-RPOTmp and anti-RPOTp, are completely specific.

Two phage-type RNA polymerases, RPOTp and RPOTmp, are localized in chloroplasts in mature spinach plants

Analysis of total, plastid and mitochondrial protein extracts by western immunoblotting reveals that both enzymes, RPOTp

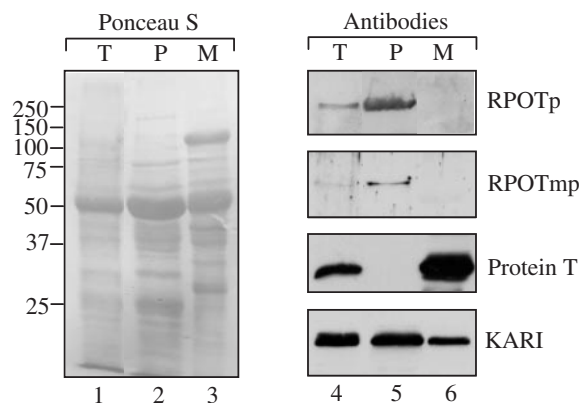


Figure 3. Both phage-type RNA polymerases, RPOTmp and RPOTp, are localized exclusively in plastids. Peptide specific IgG fractions have been obtained from the RPOTp and RPOTmp antisera as described in Materials and Methods. Equal amounts (40 μ g) of total protein extracts (lanes 1 and 4), purified chloroplasts (lanes 2 and 5) and purified mitochondria (lanes 3 and 6) have been separated by PAGE. After transfer to nitrocellulose membranes the proteins have been stained by Ponceau S (lanes 1–3) or have been analysed by immunoblotting using antisera prepared against RPOTp, RPOTmp, ProteinT and KARI (lanes 4–6).

and RPOTmp, are exclusively localized in plastids. No immunoreactive protein could be detected in mitochondria (Figure 3). The purity of the mitochondrial and plastid fractions has been verified by antibody reaction using antibodies made against KARI [acetoxy acid reductoisomerase, (36)] as plastid control and anti-ProteinT antibodies as mitochondrial control (37). These control reactions show that the mitochondrial fraction is contaminated by plastids, but, importantly, the plastid fraction is not contaminated by mitochondria, i.e. the immunoreactive RPOTp and RPOTmp proteins that are present in the chloroplast fraction do not result from mitochondrial contamination.

The two different plastid phage-type enzymes are membrane bound

Next we have analysed the sub-localization of the two NEP enzymes within plastids. To this aim, proteins of total chloroplast extract (P) have been separated together with proteins from soluble plastid extracts (S), thylakoid membranes (Tk) and envelope membranes (E) by SDS-PAGE. The corresponding fractions have been further characterized by immunoblotting using antibodies made against the plastid ribosomal protein L4 (38), the plastid inner envelope protein IE37 (39) and the membrane bound plastid terminal oxidase [PTOX, (40)] (Figure 4A). As expected, IE37 is only revealed in the envelope fraction and PTOX is only present in the thylakoid fraction thus indicating that the two membrane fractions, envelope and thylakoids, are not cross-contaminated. The r-protein L4, which has been shown recently to co-purify with the spinach T7-like transcription complex (41), is present in all fractions. The presence of L4 in the two membrane fractions might reflect the presence of membrane bound ribosomes and/or it might also reflect co-purification with at least one of the phage-type enzymes since analyses of the same fractions using the antibodies prepared against RPOTmp and RPOTp show that both enzymes are also present

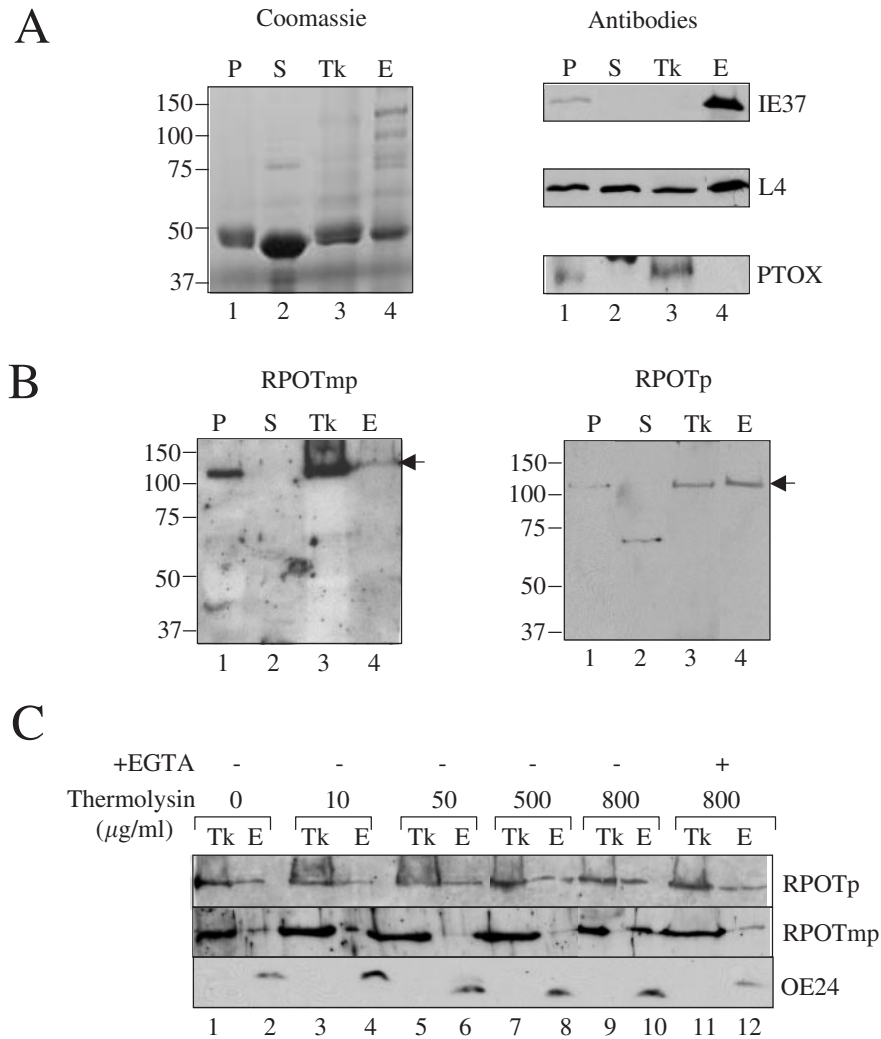


Figure 4. RPOTmp and RPOTp are bound to plastid thylakoid and envelope membranes. Aliquots (30 μg) of total chloroplast proteins (P), soluble chloroplast proteins (S), proteins obtained from purified thylakoid (Tk) and envelope (E) membranes have been separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were analysed either (A) by staining with Coomassie (left panel) or by antibodies raised against plastid inner envelope protein IE37 (IE 37) plastid ribosomal protein L4 (L4) and plastid terminal oxidase (PTOX) (right panel) or (B) by purified anti-RPOTmp IgG (Figure 3B, left panel) and by purified anti-RPOTp IgG fractions (Figure 3B, right panel). (C) Aliquots of purified intact chloroplasts have been treated either with buffer (Co, lanes 1 and 2) or with different concentrations of thermolysin (lanes 3–12) in the absence (lanes 3–10) or in the presence (lanes 11 and 12) of EGTA (Materials and Methods). After purification of thylakoid and envelope fractions equal amounts of protein (30 and 15 μg , respectively) have been analysed by immunoblotting using antibodies made against RPOTp, RPOTmp and outer envelope protein 24 (OE24).

in thylakoids as well as envelopes (Figure 4B). The two phage-type enzymes are not present in the soluble protein fraction where the presence of L4 reflects probably the presence of ribosomes.

In order to verify whether the envelope-bound enzymes correspond to not yet imported precursor protein we performed thermolysin treatment of isolated plastids. After thermolysin digestion thylakoid and envelope fractions were analysed by western immunoblotting using RPOTp and RPOTmp specific antibodies (Figure 4C). Thermolysin is active in the range of 50–800 $\mu\text{g/ml}$ as shown by the shortening of outer envelope protein 24 (lanes 6, 8 and 10; OE24) (30) and the addition of EGTA inhibits the cleavage reaction (lane 12). The two phage-type RNA polymerases, RPOTp and RPOTmp, do not disappear in the envelope fraction after treatment with thermolysin

indicating that both enzymes are positioned at the inner face of the envelope and do not correspond to precursor proteins.

Membrane binding of the two NEP enzymes is not mediated via DNA

Having determined membrane localization of the two plastid phage-type enzymes we wanted to know whether the enzymes are localized to the membranes via the plastid DNA which is known to be attached to membranes. To answer this question we performed different DNase treatments of plastid membrane fractions (Figure 5). Different DNase concentrations have been used which all digest plastid DNA completely as shown by ethidium bromide staining after separation on agarose gels (Figure 5C, ptDNA). None of the three DNase

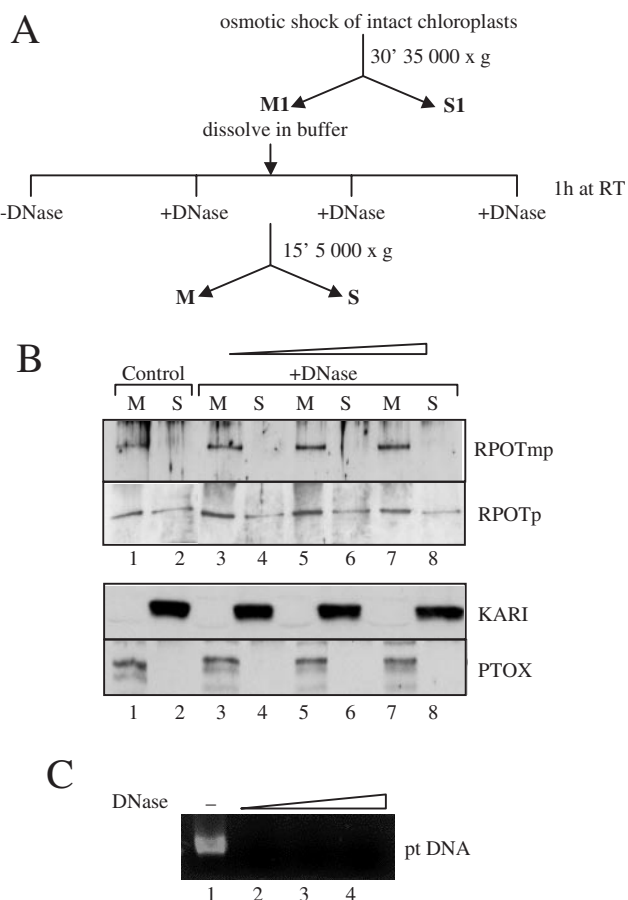


Figure 5. DNase treatment of purified plastid membrane fractions. (A) Schematic representation of the experiment. (B) Aliquots of fraction M1 corresponding to 60 µg of chlorophyll (A) have been re-suspended in low salt buffer. The solubilized membranes have been kept at room temperature for 1 h in the absence (–DNase, lanes 1 and 2) or presence (+DNase) of 7.5 µg (lanes 3 and 4), 15 µg (lanes 5 and 6) or 30 µg (lanes 7 and 8) of DNase before separating into membrane (M) and soluble (S) fractions by centrifugation. After separation of proteins by SDS–PAGE all fractions have been analysed using purified RPOtMp and RPOtP antibodies and antibodies made against the soluble plastid KARI and the membrane bound plastid terminal oxidase (PTOX). (C) DNase activity was verified by analysing the membrane fractions [M1, M3, M5 and M7, (A)] without (–, lane 1) and after DNase treatment (7.5, 15 and 30 µg of DNase, lanes 2–4, respectively) on agarose gels and staining the DNA by ethidium bromide.

concentrations used influence the membrane localization of the two NEP enzymes (Figure 5B, RPOtMp and RPOtP, upper panel) indicating that their membrane binding is not mediated via DNA. However, in contrast to RPOtMp that remains completely attached to the membrane fraction during the 1 h incubation period, RPOtP solubilizes partly from the membranes. As controls for the soluble and the membrane fractions, the stromal ketol-acid reductoisomerase (KARI) (36) and the membranous terminal oxidase (PTOX) (40) have been analysed by antibody reaction (Figure 5B, lower panel).

DISCUSSION

The existence in chloroplasts of a monomeric RNA polymerase that could be of the phage-type has been first demonstrated

in 1993 (42). Later on, the cloning of cDNAs coding for phage-type enzymes confirmed their existence in monocotyledon as well as dicotyledon plants (4–11,43,44). But the function of the phage-type enzyme(s) in plastids is not yet clear. It is believed that phage-type enzymes are especially important during early phases of chloroplast differentiation and PEP represents the principal transcriptional activity in mature chloroplasts (45,46). Although NEP as well as PEP enzymes are present in mature chloroplasts (20,47), specific NEP transcriptional activity has not yet been isolated from mature chloroplasts (20) and transcripts, corresponding to transcription initiation on NEP promoters, are present only at a very low level in photosynthetically active tissues. Therefore, many NEP promoters have been mapped using either plant material in which the PEP transcription system is not functioning or largely reduced [for a review see (48)] or using *in vitro* transcription systems that have been established from PEP deficient plant material (49,50). The down-regulation of NEP transcriptional activity during chloroplast biogenesis might be due to direct binding of glutamyl-tRNA to the enzyme (51). However, the biological meaning of this down-regulation is not yet clear and the question of the function of phage-type enzymes in mature chloroplasts remains unanswered.

As a first step to characterize phage-type enzymes from green plant tissues biochemically we have produced specific antibodies against two spinach phage-type enzymes that should correspond to RPOtP and RPOtMp enzymes as revealed by amino acid sequence alignment of hitherto known phage-type enzymes (Figure 1). By using these antibodies we could demonstrate that both enzymes are present in mature chloroplasts. Regarding the contradiction concerning plastid localization of *Arabidopsis* RPOtMp that has been raised from the papers of Hedtke *et al.* (10) and Kabeya and Sato (12) our experiments confirm plastid localization of RPOtMp in mature leaf chloroplasts of higher plants (Figure 3). Whether the localization of RPOtMp in plastids varies between different higher plant species or whether the use of GFP fusion constructs to locate proteins might give unpredictable artefacts needs further investigation. Also, the fact that we could not detect RPOtMp in mitochondria does not exclude that the enzyme might be present in mitochondria in other developmental stages. However, at least one phage-type enzyme should be always present in mitochondria. Therefore, we suggest that a third enzyme, corresponding to RPOtM, should exist in spinach. The corresponding mRNA might be rare compared with *RpoTp* and *RpoTmp* since we could not isolate a third cDNA from the cDNA library that had been prepared from actively growing leaves (Lamda ZAP®II Library; Stratagene).

Sub-fractionation of purified spinach chloroplasts show further that both phage-type enzymes are membrane bound. They are associated to thylakoid membranes as well as to envelope membranes (Figure 4A and B). Envelope localization could correspond to un-cleaved precursor proteins that have not yet been imported into the plastid. However, the analysis of thylakoid and envelope fractions, isolated after thermolysin digestion of purified intact chloroplast, shows that the two envelope-associated RNA polymerases are not subject to digestion (Figure 4C). From this result we can conclude that RPOtP and RPOtMp are bound to the inner

face of the plastid envelope. To elucidate the functional implication(s) of either envelope or thylakoid membrane binding of the two phage-type RNA polymerases represents a challenge for future studies.

The affinity of the two different phage-type enzymes to the membranes seems to be different. While RPOTmp remains associated to the plastid membranes during a 1 h incubation period at room temperature, RPOTp solubilizes partly (Figure 5B). This difference might be used in further experiments to separate the two different enzymes for *in vitro* transcription studies. The question of how the two enzymes are attached to the membranes cannot be answered yet. Bioinformatic analyses of the amino acid sequences do not reveal transmembrane domains or hydrophobic motives that could indicate direct membrane binding of the enzymes. On the contrary to what was believed previously, the localization of the two phage-type enzymes to the membrane fraction is not mediated via template DNA. This could be shown by DNase treatment of the membrane fractions (Figure 5). From this result the question raises of whether these membrane bound NEP enzymes are engaged in transcription complexes in mature chloroplasts. Since NEP initiated transcripts are of very low abundance in mature chloroplasts, it is likely to assume that NEP enzymes are sequestered to membranes for (or after) inactivation.

Studies to analyse in which way the two phage-type enzymes are attached to the thylakoid membranes and whether they are transcriptionally active are currently under way in our laboratory.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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