Intraplastidial trafficking of a phage-type RNA polymerase is mediated by a thylakoid RING-H2 protein

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The plastid genome of dicotyledonous plants is transcribed by three different RNA polymerases; an eubacterial-type enzyme, PEP; and two phage-type enzymes, RPOTp and RPOTmp. RPOTp plays an important role in chloroplast transcription, biogenesis, and mesophyll cell proliferation. RPOTmp fulfills a specific function in the transcription of the *rrn* operon in proplasts/amyloplasts during seed imbibition/germination and a more general function in chloroplasts during later developmental stages. In chloroplasts, RPOTmp is tightly associated with thylakoid membranes indicating that functional switching of RPOTmp is connected to thylakoid association. By using the yeast two-hybrid system, we have identified two proteins that interact with RPOTmp. The two proteins are very similar, both characterized by three N-terminal transmembrane domains and a C-terminal RING domain. We show that at least one of these proteins is an intrinsic thylakoid membrane protein that fixes RPOTmp on the stromal side of the thylakoid membrane, probably via the RING domain. A model is presented in which light by triggering the synthesis of the RING protein determines membrane association and functional switching of RPOTmp.

chloroplast | RING finger

Transcription of this small organellar genome is accomplished by three different RNA polymerases in a developmentally regulated manner (1, 2). One of the three RNA polymerases is of the eubacterial type, and its subunits are encoded on the plastid genome [plastid-encoded RNA polymerase (PEP)]. The two other RNA polymerases are nucleus-encoded [nucleus encoded plastid RNA polymerase (NEP)] and of the phage type (3, 4). Three different genes have been identified in *Arabidopsis* coding for NEP proteins that are localized in mitochondria (*RPOTm*, At1g68990), plastids (*RPOTp*, At2g24120), or in both organelles (*RPOTmp*, At5g15700; refs. 5 and 6). However, only two genes (*RPOTp* and *RPOTm*) have been detected in monocotyledons (4, 7, 8), thus raising the question of the function of RPOTmp in plastids of dicotyledon plants. Recent results have shown that RPOTp can be regarded as the principal NEP enzyme in chloroplasts playing an important role in chloroplast transcription, biogenesis, and mesophyll cell proliferation (9, 10). Results obtained with RPOTmp indicate partial replacement of RPOTp in *RPOTp* mutants (10, 11), an early function in light-induced accumulation of several plastid mRNAs (12, 13), and specific transcription of the *rrn* operon of *Arabidopsis* from the PC promoter during seed imbibition and germination (10).

Most of the plastid transcription units are preceded by NEP as well as PEP promoters and could be transcribed by the two types of RNA polymerase. However, with a few exceptions, NEP transcripts are barely detectable in mature chloroplasts, and most of the so-far-determined NEP promoters have been analyzed in PEP-deficient photosynthetically inactive plant material (14–17). These results led to an initial model of plastid transcription attributing special importance to NEP for the transcription of housekeeping genes during early plant development and further suggesting that PEP represents the major RNA polymerase transcribing preferentially photosynthesis related genes during later developmental stages. This model has recently been challenged by the discovery that both RNA polymerases, NEP and PEP, are already present in dry seeds of *Arabidopsis* (18). Furthermore, NEP enzymes are present in mature chloroplasts (3, 19) when NEP transcripts are rare, thus indicating a general switch in the regulation of the plastid NEP transcriptional activities during chloroplast differentiation. Two different models have recently been proposed to explain developmental switching from principally NEP to principally PEP transcripts in plastids. One of them is based on a dual function of $tRNA^{Glu}$ as precursor for chlorophyll biosynthesis and inhibitor of NEP activity. It is suggested that, during plant development and plastid differentiation, $tRNA^{Glu}$ is among the early genes transcribed by PEP, and once $tRNA^{Glu}$ is made, it will associate with NEP (RPOTp) and inactivate NEP activity (20). The other model is based on the observation that all plastid genes are transcribed in PEP-deficient tobacco plants (21) and suggests development-dependent changes in NEP and PEP mRNA turnover rates (22). However, both of these models concern only RPOTp, and it is still unclear how the activity of RPOTmp is regulated.

We have recently shown that RPOTmp is tightly attached to thylakoid membranes in spinach chloroplasts (23). This membrane attachment is not mediated via DNA, i.e., the enzyme should have an intrinsic affinity to membranes, or membrane association should occur via other proteins. In the present paper,

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we have characterized the mechanisms that lead to the strong thylakoid binding of RPOTmp. We have identified NEP interacting proteins (NIPs) of RPOTmp from mature chloroplasts. These proteins are integral membrane proteins harboring a ring finger domain that is exposed to the stromal surface of the thylakoid membrane system. From our data, we present a third model to explain developmental switching from principally NEP to principally PEP transcription. In this model, fixation of RPOTmp to thylakoids is mediated by light via light-dependent expression of NIPs and leads finally to down-regulation of plastid *rrn* transcription.

Results

Selection of Two Different NIP cDNAs by Screening of an *Arabidopsis* Two-Hybrid cDNA Library with RPOTmp-GAL4. To identify protein partner(s) that play a role in the observed strong fixation of RPOTmp to thylakoid membranes, we used the yeast two-hybrid system (Clontech). The analysis of three million cotransformants using *SoRpoTmp* (Y18853) fused to the DNA-binding domain of GAL4 gave rise to two cDNA clones (AJ400897 and AJ400898) encoding two different but highly homologous proteins of ≈25 kDa. The corresponding proteins were named AtNIP1 and AtNIP2, respectively (NIP, for NEP-interacting protein). A third cDNA (AM883105) has been isolated from a spinach library by screening with the AtNIP1 cDNA clone. The corresponding protein was named SoNIP. The alignment of the three NIPs (http://npsa-pbil.ibcp.fr) shows a high sequence identity of these proteins (Fig. 1*A*). All three proteins harbor three N-terminal transmembrane domains and a C-terminal C3H2C3 type RING finger signature (http://expasy.org/prosite). The positions of these domains are underlined (Fig. 1*A*). Transit peptide prediction by several programs like Psort (http://psort.nibb.ac.ip), ChloroP (www.cbs.dtu.dk/service/ChloroP), TargetP (www.cbs.dtu.dk/services/TargetP), and Predotar (http://urgi.versailles.inra.fr/predotar) indicated chloroplast localization for AtNIP2. For NIP1 and SoNIP, the predictions are less affirmative. NIP1 might be located in mitochondria (Psort) and SoNIP in chloroplasts (ChloroP). Plastid localization of at least one of the NIPs (NIP1 or NIP2) was confirmed by Western immunoblotting using NIP1-specific peptide antibodies that cross-react with NIP2 and SoNIP [supporting information (SI) Fig. S1]. The recent classification of all *Arabidopsis* RING finger proteins according to their RING finger domain structure groups the two NIPs into cluster 2.1 reminiscent of the RING-H2 pattern (24).

Tissue-Specific and Light-Dependent Expression of NIP. Western blot analyses of protein extracts obtained from light-exposed organs like siliques, leaves, flowers, and stems against protein extracts prepared from roots and young plantlets grown in darkness show that NIPs are present only in the light-exposed tissues (Fig. 1*B*). Light-dependent NIP expression was confirmed by analyses of proteins prepared from 7-d-old light-grown (16 h/8 h light–dark cycle) and dark-grown *Arabidopsis* plantlets (Fig. 1*C*, lanes 1 and 2). Once built up, NIPs seem to be relatively stable. No diminution of quantity is observed after a 16-h dark period (Fig. 1*C*, lane 3). On the other hand, 16-h light treatment of etiolated plantlets is not sufficient to reach the NIP quantity that is present in plants grown under the light/dark cycle (Fig. 1*C*, lane 4).

Intraplastidial Localization of NIP. Because our antibodies do not distinguish between NIP1 and NIP2, in the following, we will speak about NIP in general. Next, we determined the intraplastidial localization of NIP in the two species, spinach and *Arabidopsis*. Spinach RPOTmp was observed mainly in the thylakoid membrane fraction and to a small extent also in the envelope (23). We examined these two fractions separately for NIP localization (Fig. 2*A*). Antibodies made against the plastid inner

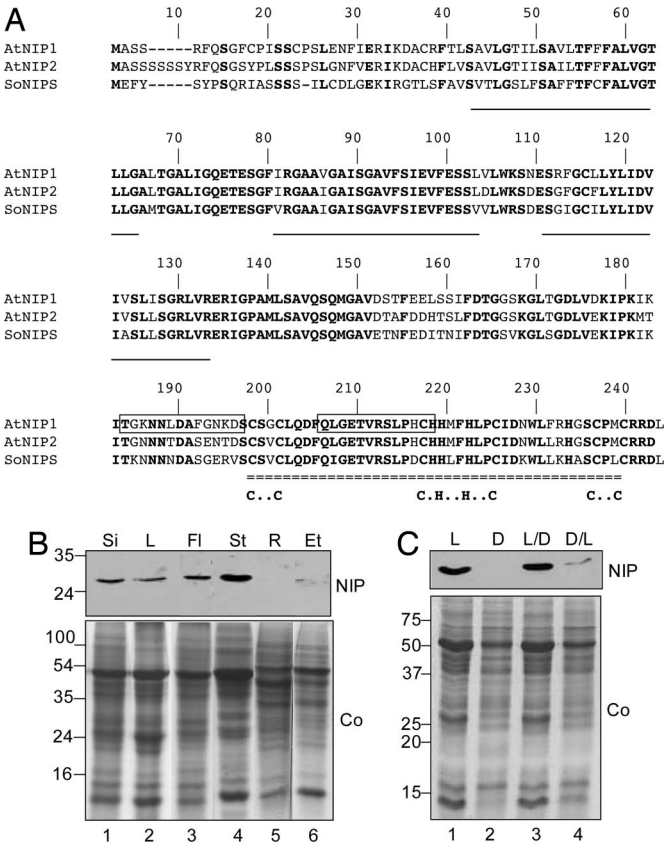

Fig. 1. NIPs are small proteins characterized by light-dependent expression. (*A*) Alignment of NIP1 (GenBank accession no. At2g17750), NIP2 (GenBank accession no. At2g17730), and SoNIP (GenBank accession no. AM883105). Identical residues are in bold. Simple bars below the sequences indicate the location of the three N-terminal transmembrane domains. The C-terminal RING domain is double-underlined, and the significant amino acids that determine the RING are noted below the line. The two peptides that have been used for antibody production are boxed. (*B*) Tissue-specific expression of NIP. Thirty micrograms of proteins prepared from *Arabidopsis* siliques (lane 1), leaves (lane 2), flowers (lane 3), stems (lane 4), roots (lane 5), and etioplated seedlings (lane 6) were separated by SDS/PAGE and either stained by Coomassie (Co, *Lower*) or analyzed by NIP antibodies after transfer to Nylon membranes (NIP, *Upper*). (*C*) Light-dependent expression of NIP. Total proteins from 7-day-old *Arabidopsis* seedling were analyzed by Western immunoblotting by using NIP1 antibodies (*Upper*). Plantlets were grown for 7 days in light (lane 1) or dark (lane 2) or 6-day-old light- (lane 3) or dark-grown (lane 4) plantlets were transferred for the last 24 h either in darkness (lane 3) or to light (lane 4). *Lower* shows the Coomassie staining of the SDS gel as loading control. Molecular mass markers are indicated at the left.

envelope protein IE37 (25), RpL4 (26), and D1 protein (PsbA, Agrisera) served as controls. The IE-37 and D1 antibody reactions show that the soluble (S), envelope (E), and thylakoid (Tk) fractions are not cross-contaminated. R-protein L4 is detected in all fractions, i.e., crude membranes (M), supernatant (S), total plastids (P), envelope (E), and, in traces, in thylakoids (Tk). NIP is attached exclusively to the thylakoids. No NIP is detected in the envelope fraction. The same holds true for the corresponding *Arabidopsis* plastid subfractions (Fig. 2*B*).

Membrane Topology of NIP. To test whether NIP is an integral membrane protein, purified thylakoid membranes have been washed either with buffer only or with buffer supplemented with 1 M NaCl or with 0.1 M NaOH (Fig. 2*C*). Afterward, membrane proteins have been analyzed with antibodies made against RpL4, against the 33-kDa protein of the oxygen-evolving complex

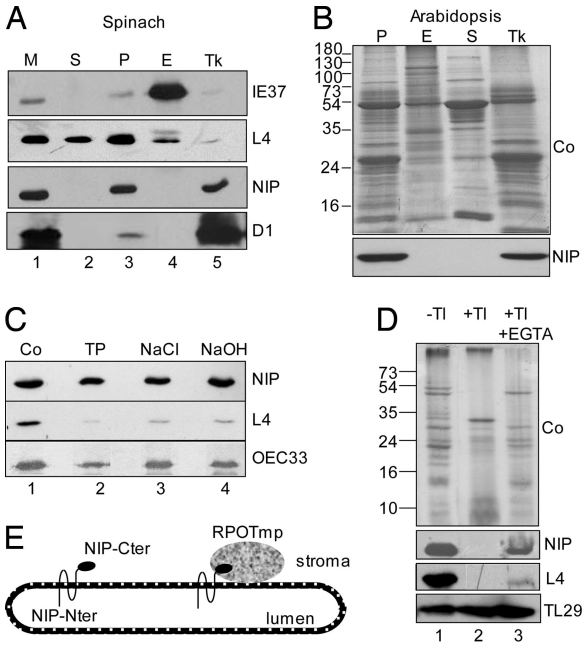

Fig. 2. NIPs are integral thylakoid membrane proteins. NIPs are associated with thylakoid membranes. (*A*) Spinach chloroplasts (lane 3) were fractionated either into crude membrane (lane 1) and soluble (lane 2) fractions or into envelope (lane 4) and thylakoid (lane 5) membranes. Equal amounts of protein have been separated by SDS/PAGE and analyzed by immunoblotting by using antibodies made against inner envelope protein 37 (IE37), plastid ribosomal protein L4, NIP1, and D1 protein. (*B*) *Arabidosis* chloroplasts (lane 1) have been further fractionated into envelope (lane 2), soluble (lane 3), and thylakoid (lane 4) fractions, and proteins have been analyzed by Western immunoblotting by using NIP1 antibodies (*Lower*). The Coomassie-stained polyacrylamide gel is shown as loading control (*Upper*). Molecular-mass markers are indicated at the left. (*C*) Either thylakoid membrane proteins were analyzed directly (lanes 1), or membranes have been washed with buffer (lane 2), 1 M NaCl (lane 3), or 0.1 M NaOH before protein extraction and Western immunoblotting using NIP1 antibodies (*Top*), antibodies against r-protein L4 (*Middle*) as negativ control and antibodies against a protein of the oxygen evolving complex (OEC33, *Bottom*) as positive control. (*D*) Thermolysin treatment on intact spinach thylakoids. Twenty micrograms of proteins of untreated thylakoids (lane 1) or thylakoids treated with thermolysin either in the absence (lane 2) or in the presence of 20 mM EGTA as specific inhibitor (lane 3) were analyzed by Western immunoblotting by using NIP antibodies (NIP), L4 antibodies (L4), and TL29 antibodies as control for a thylakoid lumen protein (TL29). *Upper* shows the polypeptide pattern of the different fractions after Commassie staining (Co). Molecular-mass markers are indicated at the left. (*E*) Schematic representation of NIP-RPOTmp localization on the thylakoid membranes.

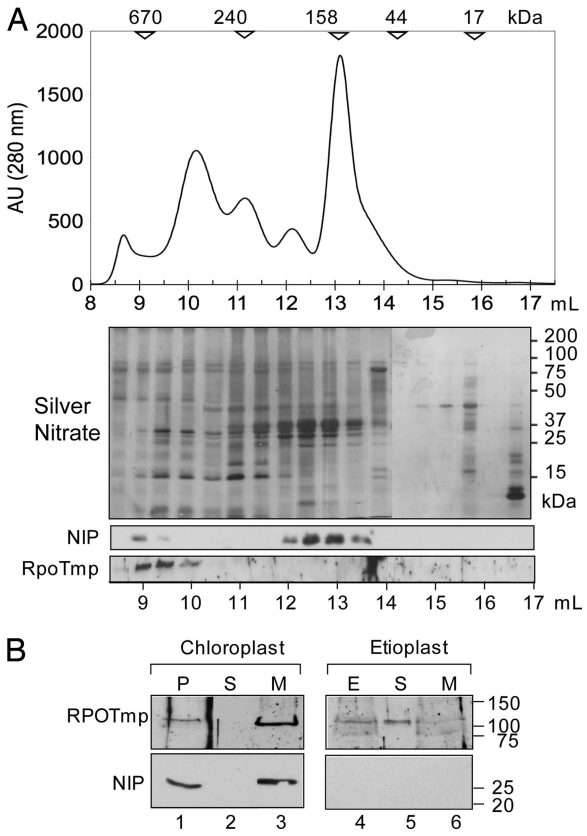

Fig. 3. Confirmation of NIP-RPOTmp interactions. (*A*) Fractionation of thylakoid-associated proteins on Superose 6 HR10/30 column after solubilization by *N*-dodecyl lauryl maltoside (Sigma). *Top* shows the elution profile. *Middle* corresponds to the protein pattern of each fraction as revealed by silver staining, and *Bottom* represents Western immunoblotting of each fraction by using antibodies against NIP1 and RPOTmp. (*B*) Immunolocalization of NIP and RPOTmp in protein extracts of spinach chloroplast and etioplast. Chloroplast proteins (30 μg) (P, lanes 1–3) and etioplast proteins (E, lanes 4–6) were analyzed directly (lanes 1 and 4) or after further fractionation into soluble (S, lanes 2 and 5) and membrane (M, lanes 3 and 6) fractions. After separation by SDS/PAGE, proteins were transferred to Nitrocellulose membranes, and the blot was analyzed by using antibodies produced against SoRPOTmp (*Upper*) and NIP1 (*Lower*). Molecular-mass markers are indicated at the right.

(OEC33; ref. 27), and against NIP. All three washing procedures detach RpL4 from the membranes, as expected for a protein that sticks nonspecifically to the thylakoids' stromal surface. The OEC33 polypeptide that is localized on the luminal face of the thylakoid membrane does not diminish, and also NIP remains associated to the thylakoids even after washing with NaCl and NaOH. This shows that NIP is an integral membrane protein.

The question of whether the C-terminal ring finger domain of NIP is exposed to the lumen or to the stromal side of the thylakoids was addressed by Western immunoblotting of purified thylakoid fractions after treatment with thermolysin (Fig. 2*D*). The antibodies are made against a peptide that is located in the C-terminal protein part. If the C terminus is protected from thermolysin digestion, Western blot analysis should result in the appearance of a shorter protein than the full-length NIP. The r-protein L4 and the thylakoid luminal protein TL29 (28) were analyzed as controls. Both proteins, NIP and RpL4, disappear completely after protease treatment in the absence of EGTA, i.e., the C-terminal ring finger should be located on the stromal side of the thylakoids. From this result, we conclude that NIP should be integrated into the thylakoid membrane as schematically illustrated (Fig. 2*E*), and RPOTmp might be localized to the stromal side of the thylakoid membranes by interaction with the ring finger domain of NIP.

Confirmation of Interaction Between NIP and RpoTmp. Direct interaction between NIP and RPOTmp is strongly suggested by the fact that NIP has been selected by using a yeast two-hybrid screen. To confirm this interaction by another method, we have solubilized macromolecular protein complexes of the chloroplast thylakoid membranes by mild detergent treatment (see *Materials and Methods*), and we have analyzed these complexes by gel filtration on a Superdex 200 HR10/30 column. Western immunoblot analysis of all Superdex fractions shows elution of NIP in protein complexes of two different sizes, one corresponding to a high-molecular-mass complex larger than 670 kDa and the other one to lower-molecular-mass complexes in the range of 150 kDa. RPOTmp coelutes with the high-molecular-mass complex (Fig. 3*A*). None of the two proteins is found in fractions corresponding to their monomeric forms. This result suggests

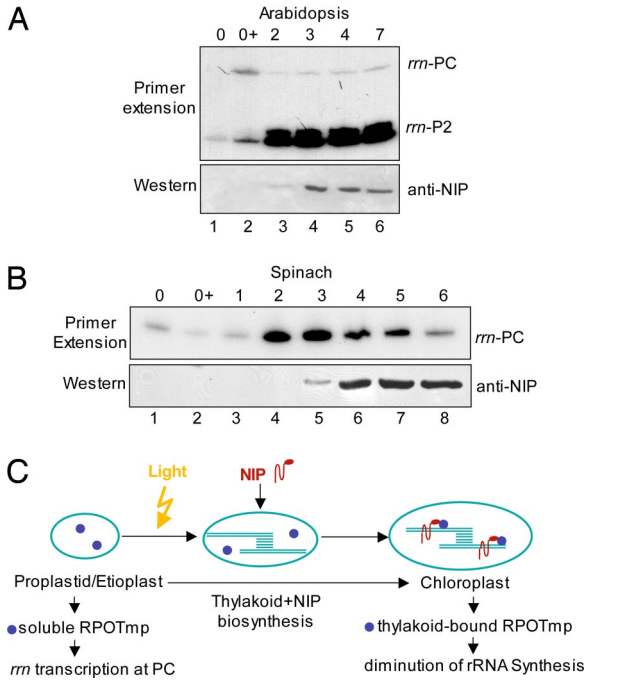

Fig. 4. NIP expression correlates with reduction of *rrn*-PC transcript levels. (*A*) 16S rRNA precursor transcripts have been analyzed by primer extension (*Upper*) by using total RNA of *Arabidopsis* dry seeds (lane 1), seeds after imbibition (lane 2), and seedling 2, 3, 4, and 7 day after the beginning of germination (lanes 3–6). Total proteins have been isolated from the same plant material and analyzed by Western immunoblotting by using NIP1 antibodies (*Lower*). (*B*) 16S rRNA precursor transcripts have been analyzed by primer extension (*Upper*) by using total RNA from spinach dry seeds (lane 1), seeds after imbibition (lane 2), and seedling 2, 3, 4, and 7 day after the beginning of germination (lanes 3–6). Total proteins have been isolated from the same plant material and analyzed by Western immunoblotting by using NIP1 antibodies (*Lower*). (*C*) Model of NIP and RPOTmp production and interaction during chloroplast differentiation.

that both proteins exist in a very large complex together with additional proteins that are not yet identified or as multidimers comprising several NIP and RPOTmp molecules. Although we do not know yet the exact composition of this complex, the result confirms interaction between NIP and RPOTmp.

Finally, if RPOTmp is fixed to the thylakoid membranes by NIP as suggested, RPOTmp should be soluble in plastids that do not contain NIP, e.g., in etioplasts of dark-grown plants. To test this hypothesis, we have isolated chloroplasts and etioplasts from light- and dark-grown spinach plantlets, respectively, and we have analyzed the soluble and the membrane fractions by immunoblotting using antibodies against RPOTmp and NIP (Fig. 3*B*). In NIP-containing chloroplasts, RPOTmp sediments with the membrane fraction (Fig. 3*B*, lane 3), whereas in NIP-deficient etioplasts, RPOTmp remains in the soluble fraction (Fig. 3*B*, lane 5).

The Appearance of NIP Coincides with a Reduction of 16S rRNA Transcripts Initiated at PC. We have recently shown that RpoTmp transcribes specifically the *rrn* operon at the PC promoter during imbibition of *Arabidopsis* seeds (10), i.e., during a developmental period when thykoids are not yet established. After transfer of the seeds to light the amount of PC initiated transcripts drop down, although the RPOTmp protein level augments until 2 days after germination and afterward remains constant until day six (18). Now, we have analyzed PC transcript levels by primer extension and NIP protein levels by immunoblotting during germination and early seedling development (Fig. 4*A*). The result shows that the appearance of NIP correlates with the diminution of PC transcripts. This suggests that high-level *rrn* transcription at PC is performed by soluble RPOTmp in non-photosynthetic plastids. After illumination, when NIP is made up along with the thylakoid membrane system, RPOTmp is placed to the membrane and *rrn* transcription diminishes.

In contrast to *Arabidopsis*, where the *rrn* operon is transcribed from two different promoters, PC and P2 (29), in spinach, the operon is exclusively transcribed from the PC promoter (30–32). The analysis of PC transcript levels by primer extension and NIP protein levels by immunoblotting during germination and early seedling development of spinach shows the same correlation as found for *Arabidopsis*. PC-initiated transcripts diminish when NIP protein appears (Fig. 4*B*). RPOTmp protein levels could not be analyzed during spinach germination, because our antibody is not strong enough to reveal the protein in total protein extracts during this developmental period (18).

Discussion

Recently, we have shown that the plastid phage-type RNA polymerase RPOTmp transcribes specifically the *rrn* operon at the PC promoter, and in this way is distinct from the second plastid phage-type RNA polymerase RPOTp (10). To further characterize the way of functioning of this phage-type RNA polymerase, we have used the yeast two-hybrid system to clone cDNAs encoding RPOTmp interacting proteins. A high-throughput screen yielded two clones, both coding for very similar but different proteins, named NIP1 (At2g17750) and NIP2 (At2g17730). Although two additional NIP-like proteins exist as revealed by bioinformatic research (not shown), none of these NIP-like proteins and no other protein coding clone has been selected by the two-hybrid system, indicating specificity of interaction of only NIP1 and NIP2 with RPOTmp.

Alignment of NIP1, NIP2, and NIPS (Fig. 1*A*) shows high similarity of the three proteins except in the N-terminal parts representing putative transit peptides. Bioinformatic analysis indicates NIP1 and NIP2 as candidates for organelle localization, either mitochondrial or plastidial. However, the presence of NIP could be demonstrated only in chloroplasts and not in mitochondria (Fig. S1). This indicates different paths of regulation of RPOTmp activity in mitochondria and chloroplasts. In fact, the function of RPOTmp in mitochondria is not yet clear, because *RPOTmp* mutants do not show major differences in mitochondrial gene transcripts (12).

All three NIPs harbor three N-terminal transmembrane helixes and a C-terminal RING domain. The presence of three transmembrane helices suggests the C-terminal regions of the proteins are located on the opposite side of the membrane than the N-terminal regions, and our results characterize NIP as an integral thylakoid membrane protein having the N-terminal RING domain oriented vs. the stromal side of the thylakoids (Fig. 2 *A–E*). RING domains belong to the most frequently detected domains in the *Arabidopsis* proteome (24). However, up to now, none of these proteins has been described in chloroplasts. In general, RING domains are known to mediate protein–protein interactions and function in the formation and architecture of large multiprotein complexes (33). In chloroplasts, the RING domain should therefore be involved in the interaction of NIP with RPOTmp, i.e., in the fixation of RPOTmp to the thylakoid membranes.

The interactions between NIP and RPOTmp are strong enough to resist the detergent treatment used to solubilize the thylakoids. Both proteins, NIP and RPOTmp, coelute from the Sepharose 6 column in fractions reminiscent of protein complexes >670 kDa (Fig. 3*A*), thus indicating the formation of a large multiprotein complex on the thylakoid surface. A further step in understanding the functioning of RPOTmp would consist in the characterization of this complex. The small NIP complexes

that do not coelute with RPOTmp (100–200 kDa) correspond probably to NIP oligomers, the formation of which can be observed by SDS/PAGE after protein cross-linking (not shown). However, we cannot exclude that NIP interacts also with other proteins and forms other complexes than NIP/RPOTmp.

Our results show that RPOTmp is soluble in nonphotosynthetic cells where NIP does not exist and becomes membrane-attached during light-triggered cell differentiation as soon as thylakoid membranes are established and NIP accumulates (Figs. 1 *A* and *B* and 3*B*). This opens the question of whether this intraplastidial trafficking of RPOTmp is accompanied by functional changes of the RNA polymerase. As already mentioned, RPOTmp is of special importance for the transcription of the *rrn* operon at the PC promoter during early developmental stages, i.e., in yet-nonphotosynthetic cells. It is also known that *rrn* transcription is highest in proplastids and during early periods of chloroplast differentiation and rapidly drops down once mature chloroplasts are established (32). The simplest hypothesis to explain RPOTmp trafficking during chloroplast differentiation is therefore light- and development-dependent diminution of *rrn* transcription by sequestering the enzyme to the thylakoid membrane. The complementary expression pattern of *rrn*-PC transcripts and NIP quantities during germination, i.e., during plastid differentiation from nonphotosynthetic proplastids into mature chloroplasts, supports this hypothesis (Fig. 4 *A* and *B*), which is schematically shown in Fig. 4*C*. In later developmental stages, once RPOTmp is membrane-attached, it functionally overlaps with RPOTp (10, 11) but might also have additional functions related to stress responses because of changing growth conditions. Work is in progress in our laboratory to analyze the function of RPOTmp in later developmental stages in response to stress conditions.

Materials and Methods

Plant Material and Growth Conditions. After stratification at 4°C for 3 days in the dark, *Arabidopsis* plants, ecotype Columbia, were grown in growth chambers at 24°C in 16-h dark, 8-h light photoperiod (100 μmol of photons $m^{-2}{\cdot}s^{-1}$).

Spinach seeds (Spinacia oleracea cv ''Geant d'hiver'') were soaked overnight in water, and plants were grown at an 18°C in 10-h dark/14-h light cycle (60 μmol of photons $m^{-2}{\cdot}s^{-1}$).

Two-Hybrid System. The two-hybrid system was a GAL4-based system. Screening for RpoTmp interacting proteins was done by using the *Arabidopsis thaliana* MATCHMAKER cDNA library (Clontech, catalogue no. FL4000AB, lot no. 47045, for sale in 1996). This library was constructed in pGAD10 (National Center for Biotechnology Information gi 532698) and was supplied as culture in *Escherichia coli* DH5α. The bait, i.e., the spinach RpoTmp cDNA corresponding to amino acids 46–1036 (Y18852), was cloned into vector pAS2–1 (Clontech, catalogue no. K1604-B, gi 125657). Screening was done by cotransformation of the pAS2-1-RpoTmp plasmid (RpoTmp fused to the GAL4 DNA-binding domain) and the pGAD10 library plasmids (*Arabidopsis* cDNA library fused to the GAL4 transcriptional activation domain) into yeast CG-1945 according to the LiAc method (34), as indicated in the suppliers' protocol (PT1020-1, PT3247-1). Transformed cells were plated on SD medium containing adenine. Of 2×10^6 yeast transformants, 26 clones were selected as His+ colonies (supplemented with 5 mM 3AT). In a second screening, performed on SD plates containing α-galactosidase without amino acids, two clones were selected and sequenced, corresponding to AtNIP1 and AtNIP2.

Antibody Preparation and Purification. Peptide antibodies have been prepared in rabbits according to the DoubleX program of Eurogentec. Peptides are indicated in Fig. 1. Antibodies have been purified on Affi-Gel-peptide columns as described (23).

Production of Recombinant Proteins. *AtNIP1* and *AtNIP2* cDNAs devoid of the supposed transit peptide region were cloned in-frame into pET28a (Novagen) and pBAD/HisC (Invitrogen) expression vectors, respectively. The *SoNIP* cDNA was cloned into pQE30 vector (Qiagen). Recombinant AtNIP2 was produced in *E. coli* TOP10, and recombinant AtNIP1 and SoNIP were produced in *E. coli* XL1 blue according to suppliers' protocols.

Protein Analysis. Proteins have been isolated and analyzed by Western immunoblotting as described (35). After separation of equal amounts of protein by SDS/PAGE, polypeptides were either stained by Coomassie R250 or transferred to nitrocellulose (Schleicher & Schuell). Transferred proteins were analyzed by antibody reaction by using the ECL Western blot analysis detection system (Amersham Biosciences).

Gel Filtration. Chloroplast membranes were obtained after osmotic shock of intact chloroplasts in buffer A (10 mM Mops, pH 7.8; 4 mM $MgCl_2$), two washing steps, and recovery by centrifugation (30 min, 78,000 $\times g$, rotor S140 AT). Membranes were suspended in buffer A supplemented with 10% glycerol and 150 mM NaCl, and chlorophyll concentration was adjusted to 2 mg/ml. Membranes were dissolved with 1% *N*-dodecyl lauryl maltoside (Sigma) at 4°C for 30 min by gentle rotation. Insoluble material was removed by centrifugation (35,000 $\times g$, 30 min).

The solubilized membranes (400 μg of chlorophyll) were separated on a preequilibrated Superdex HR10/30 column (Amersham Pharmacia, buffer A containing 0.1% *N*-dodecyl lauryl maltoside), and protein was measured at 280 nm. Gel filtration standards (Bio-Rad) were used to calibrate the column. Proteins were precipitated with TCA (10% final) before SDS/PAGE and either stained by silver nitrate (36) or transferred onto nitrocellulose for Western blot analysis.

Chloroplast Preparation and Subfractionation. Pea chloroplasts (*Pisum sativum* cv. ''Douce provence'') were obtained according to Robinson and Barnett (37).

Spinach chloroplasts were purified by isopycnic centrifugation by using Percoll gradients (38). Stroma, thylakoids, and envelope fractions were obtained by sucrose gradient centrifugation as described in Douce *et al.* (39).

Arabidopsis chloroplasts and chloroplast subfractions were prepared as described (40). After lysis of intact chloroplasts, stroma, thylakoids, and envelope fractions were separated on a two-step sucrose gradient (0.6 M and 0.9 M sucrose in 0.3 M sorbitol, 5 mM $MgCl_2$, 2.5 mM EDTA, 20 mM Tricine/KOH, pH 7.6).

Tomato (*Lycopersicon esculentum*) chloroplast were isolated from 100 g of leaves by homogenization in 500 ml of grinding buffer (0.33 M sorbitol, 1 mM $MgCl_2$, 1 mM $Na_4P_2O_7$, 2 mM EDTA, 1 mM DTT, 50 mM Hepes/NaOH, pH 6.8), filtration through Miracloth (Calbiochem) and centrifugation (5,000 $\times g$, 15 min). The pellet was suspended in GR, and chloroplasts were fractionated on a discontinuous 40–80% Percoll gradient (5,000 $\times g$, 20 min). Intact chloroplasts, located at the interface, were washed, pelleted (5,000 $\times g$, 10 min), and suspended in GR.

Thermolysin Treatment of Thylakoids. Intact spinach thylakoids were treated with thermolysin from *Bacillus thermoproteolyticus* (Boehringer). Thylakoids were suspended in 10 mM 4-morpholinepropanesulfonic acid (MOPS) (pH 7.8), 4 mM $MgCl_2$, 10 mM $CaCl_2$, and 0.3 M sorbitol and incubated with thermolysin (800 μg/ml, Boehringer) at 4°C for 1h. Thermolysin treatment was stopped by addition of 20 mM EGTA, and thylakoids were sedimented (5,000 $\times g$, 15 min) and suspended in 10 mM MOPS (pH 7.8), 4 mM $MgCl_2$.

Preparation of Etioplasts and Subfractionation. Etioplasts were purified from 12-day-old dark-grown spinach seedlings according to Krause *et al.* (41) Briefly, etiolated seedlings were homogenized in buffer A [0.33 M sorbitol, 50 mM Hepes, 0.8 mM $MgCl_2$, 4 mM EDTA, 1 mM KH_2PO_4, 0.2% (wt/vol) BSA, pH 7.8], filtered through Miracloth (Calbiochem), and centrifuged (5,000 $\times g$, 15 min). The pellet was suspended in buffer B (0.33 M sorbitol, 50 mM Hepes, 1 mM EDTA, pH 7.8), and loaded on a 30–80% discontinuous Percoll gradient. Intact etioplasts were collected at interface after centrifugation (5,000 $\times g$, 25 min). Etioplasts were washed, suspended in buffer B, and lysed in 10 volumes of 10 mM MOPS, 4 mM $MgCl_2$, 1 mM PMSF (pH 7.8). Soluble and membrane fractions were finally separated by centrifugation (35,000 $\times g$, 20 min).

Preparation of Mitochondria. All procedures were done at 0–5°C. Mitochondria were prepared from potato tubers or from 3- to 4- week-old *Arabidopsis* plants according to Werhahn *et al.* (42), Kruft *et al.* (43), and Gualberto *et al.* (44).

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1. Shiina T, Tsunoyama Y, Nakahira Y, Khan MS (2005) Plastid RNA polymerase, promoters, and transcription regulators in higher plants. *Int Rev Cytol* 244:1–68.
2. Liere K, Börner T (2006) in *Regulation of Transcription in Plants*, ed Grasser KD (Blackwell, Boston), pp 184–222.
3. Lerbs-Mache S (1993) The 110-kDa polypeptide of spinach plastid DNA-dependent RNA polymerase: Single-subunit enzyme or catalytic core of multimeric enzyme complexes? *Proc Natl Acad Sci USA* 90:5509–5513.
4. Chang CC, Sheen J, Niwa Y, Lerbs-Mache S, Stern DB (1999) Functional analysis of two Maize cDNAs encoding T7-like RNA polymerases. *Plant Cell* 11:911–926.
5. Hedtke B, Börner T, Weihe A (1997) Mitochondrial and chloroplast phage-type RNA polymerases in *Arabidopsis*. *Science* 277:809–811.
6. Hedtke B, Börner T, Weihe A (2000) One RNA polymerase serving two genomes. *EMBO Rep* 1:435–440.
7. Ikeda TM, Gray MW (1999) Identification and characterization of T3/T7 bacteriophage-like RNA polymerase sequences in wheat. *Plant Mol Biol* 40:567–578.
8. Emanuel C, Weihe A, Graner A, Hess WR, Börner T (2004) Chloroplast development affects expression of phage-type RNA polymerases in barley leaves. *Plant J* 38:460–472.
9. Hricova A, Quesada V, Micol JL (2006) The *SCABRA3* nuclear gene encodes the plastid RpoTp RNA polymerase, which is required for chloroplast biogenesis and mesophyll cell proliferation in Arabidopsis. *Plant Physiol* 141:942–956.
10. Courtois F, Merendino L, Demarsy E, Mache R, Lerbs-Mache S (2007) Phage type RNA polymerase RPOTmp transcribes the *rrn* operon from the PC promoter at early developmental stages in Arabidopsis. *Plant Physiol* 145:712–721.
11. Swiatecka-Hagenbruch M, Emanuel C, Hedtke B, Liere K, Börner T (2008) Impaired function of the phage-type RNA polymerase RpoTp in transcription of chloroplast genes is compensated by a second phage-type RNA polymerase. *Nucleic Acids Res* 36:785–792.
12. Baba K, *et al*. (2004) Organellar gene transcription and early seedling development are affected in the *rpoT;2* mutant of *Arabidopsis*. *Plant J* 38:38–48.
13. Emanuel C, von Groll U, Müller M, Börner T, Weihe A (2006) Development- and tissue-specific expression of the *RpoT* gene family of *Arabidopsis* encoding mitochondrial and plastid RNA polymerase. *Planta* 223:998–1009.
14. Hajdukiewicz PTJ, Allison LA, Maliga P (1997) The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. *EMBO J* 16:4041–14048.
15. Hübschmann T, Börner T (1998) Characterisation of transcript initiation sites in ribosome-deficient barley plastids. *Plant Mol Biol* 36:493–496.
16. Silhavy D, Maliga P (1998) Mapping of the promoters for the nucleus-encoded plastid RNA polymerase (NEP) in the iojap maize mutant. *Curr Genet* 33:340–344.
17. Swiatecka-Hagenbruch M, Liere K, Börner T (2007) High diversity of plastid promoters in *Arabidopsis thaliana*. *Mol Genet Genomics* 277:725–734.
18. Demarsy E, Courtois F, Azevedo J, Buhot L, Lerbs-Mache S (2006) Building up of the plastid transcriptional machinery during germination and early plant development. *Plant Physiol* 142:993–10003.
19. Bligny M, *et al*. (2000) Regulation of plastid rDNA transcription by interaction of CDF2 with two different RNA polymerases. *EMBO J* 19:1851–1860.
20. Hanaoka M, Kanamaru K, Fujiwara M, Takahashi H, Tanaka K (2005) Glutamyl-tRNA mediates a switch in RNA polymerase use during chloroplast biogenesis. *EMBO Rep* 6:545–550.
21. Legen J, *et al*. (2002) Comparative analysis of plastid transcription profiles of entire plastid chromosomes from tobacco attributed to wild-type and PEP-deficient transcription machineries. *Plant J* 31:171–188.
22. Cahoon AB, Harris FM, Stern D (2004) Analysis of developing maize plastids reveals two mRNA stability classes correlating with RNA polymerase type. *EMBO Rep* 5:801–806.
23. Azevedo J, Courtois F, Lerbs-Mache S (2006) Sub-plastidial localization of two different phage-type RNA polymerases in spinach chloroplasts. *Nucleic Acids Res* 34:436–444.
24. Kosarev P, Mayer FX, Hardtke S (2002) Evaluation and classification of RING-finger domains encoded by the *Arabidopsis* genome. *Genome Biol* 3:1–12.
25. Block MA, Dorne AJ, Joyard J, Douce R (1983) Preparation and characterization of membrane fractions enriched in outer and inner envelope membranes from spinach chloroplasts. *J Biol Chem* 258:13273–13280.
26. Trifa Y, Privat I, Gagnon J, Baeza L, Lerbs-Mache S (1998) The nuclear *RPL4* gene encodes a chloroplast protein that co-purifies with the T7-like transcription complex as well as plastid ribosomes. *J Biol Chem* 273:3980–3985.
27. Hutin C, *et al*. (2003) Early light-induced proteins protect Arabidopsis from photooxidative stress. *Proc Natl Acad Sci USA* 15:4921–4926.
28. Bally J, *et al*. (2008) Both the stroma and thylakoid lumen of tobacco chloroplasts are competent for the formation of disulphide bonds in recombinant proteins. *Plant Biotchnol J* 6:46–61.
29. Pfannschmidt T, Link G (1997) The A and B forms of plastid DNA-dependent RNA polymerase from mustard (*Sinapis alba* L.) transcribe the same genes in a different developmental context. *Mol Gen Genet* 257:35–44.
30. Baeza L, Bertrand A, Mache R, Lerbs-Mache S (1991) Characterization of a protein binding sequence in the promoter region of the 16S rRNA gene of the spinach chloroplast genome. *Nucleic Acids Res* 19:3577–3581.
31. Iratni R, Baeza L, Andreeva A, Mache R, Lerbs-Mache S (1994) Regulation of rDNA transcription in chloroplasts: promoter exclusion by constitutive repression. *Genes Dev* 8:2928–2938.
32. Lerbs-Mache S (2000) Regulation of rDNA transcription in plastids of higher plants. *Biochimie* 82:525–535.
33. Borden KLB (2000) RING domains: Master builders of molecular scafforlds. *J Mol Biol* 295:1103–1112.
34. Gietz RD, Schiestl RH (1995) Transforming yeast with DNA. *Methods Mol Cell Biol* 5:255–269.
35. Privat I, Hakimi MA, Buhot L, Favory JJ, Lerbs-Mache S (2003) Characterization of *Arabidopsis* plastid sigma-like transcription factors SIG1, SIG2 and SIG3. *Plant Mol Biol* 55:385–399.
36. Swain M, Ross NW (1995) A silver stain protocol for proteins yielding high resolution and transparent background in sodium dodecylsulfate-polyacrylamide gels. *Electrophoresis* 16:948–951.
37. Robinson C, Barnett LK (1988) in *Plant Mol Biol: A Practical Approach*, ed Shaw CH (IRL Press, Washington, DC), pp 67–78.
38. Douce R, Joyard J (1982) in *Methods in Chloroplast Molecular Biology,* eds Edelman M, Hallick R, Chua NH. (Elsevier, Amsterdam), pp 239–256.
39. Douce R, Holtz RB, Benson AA (1973) Isolation and properties of the envelope of spinach chloroplasts. *J Biol Chem* 25:7215–7222.
40. Block MA, Tewari AK, Albrieux C, Maréchal E, Joyard J (2002) The plant *S*-adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase is located in both envelope and thylakoid chloroplast membranes. *Eur J Biochem* 269:240–248.
41. Krause K, Maier RM, Kofer W, Krupinska K, Herrmann RG (2000) Disruption of plastid-encoded RNA polymerase genes in tobacco: expression of only a distinct set of genes is not based on selective transcription of the plastid genome. *Mol Genet Genomics* 263:1022–1030.
42. Werhahn W, *et al*. (2001) Purification and characterization of the preprotein translocase of the outer mitochondrial membrane from Arabidopsis. Identification of multiple forms of TOM20. *Plant Physiol* 125:943–954.
43. Kruft V, Eubel H, Jansch L, Werhahn W, Braun HP (2001) Proteomic approach to identify novel mitochondrial proteins in Arabidopsis. *Plant Physiol* 127:1694–1710.
44. Gualberto JM, Wintz H, Weil JH, Grienenberger JM (1988) The genes coding for subunit 3 of NADH dehydrogenase and for ribosomal protein S12 are present in the wheat and maize mitochondrial genomes and are co-transcribed. *Mol Gen Genet* 215:118–127.