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Faithful transcription initiation from a mitochondrial promoter in transgenic plastids

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ABSTRACT

The transcriptional machineries of plastids and mitochondria in higher plants exhibit striking similarities. All mitochondrial genes and part of the plastid genes are transcribed by related phage-type RNA polymerases. Furthermore, the majority of mitochondrial promoters and a subset of plastid promoters show a similar structural organization. We show here that the plant mitochondrial *atpA* promoter is recognized by plastid RNA polymerases *in vitro* and *in vivo*. The *Arabidopsis* phage-type RNA polymerase RpoTp, an enzyme localized exclusively to plastids, was found to recognize the mitochondrial *atpA* promoter in *in vitro* assays suggesting the possibility that mitochondrial promoters might function as well in plastids. We have, therefore, generated transplastomic tobacco plants harboring in their chloroplast genome the *atpA* promoter fused to the coding region of the bacterial *nptII* gene. The chimeric *nptII* gene was found to be efficiently transcribed in chloroplasts. Mapping of the 5′ ends of the *nptII* transcripts revealed accurate recognition of the *atpA* promoter by the chloroplast transcription machinery. We show further that the 5′ untranslated region (UTR) of the mitochondrial *atpA* transcript is capable of mediating translation in chloroplasts. The functional and evolutionary implications of these findings as well as possible applications in chloroplast genome engineering are discussed.

INTRODUCTION

Mitochondria are derived from a formerly free-living α-proteobacterium, whereas plastids (chloroplasts) evolved from a free-living cyanobacterium. During evolution of the various lineages of eukaryotic organisms, mitochondrial and plastid genomes have undergone a drastic reduction in size, either by gene transfer from the organelles to the nucleus, or by outright gene loss (1–3). Metabolism and genetic apparatus of both types of organelles have retained bacterial features, but co-evolution of two (nuclear, mitochondrial) or three genomes (nuclear, mitochondrial, plastid) resulted not only in a redistribution of genes but also of gene products. As a consequence, typical eukaryotic proteins have taken over functions in the organelles by replacing the components of bacterial origin (4–6).

Interestingly, also genes and enzymes evidently originating from bacteriophage genomes play important roles in mitochondrial replication and transcription (7), among them the mitochondrial RNA polymerase which is related to RNA polymerases of phages like T3 and T7 (8,9). Plastid genomes harbor genes for the core subunits of a bacterial-type RNA polymerase inherited from the cyanobacterial ancestor. With support from one (out of several) nuclear-encoded sigma factors, this plastid-encoded core polymerase (PEP) recognizes bacterial-type promoters with conserved −10 and −35 boxes (9–11). In angiosperms, transcription of plastid genes needs an additional nuclear-encoded RNA polymerase (NEP) of the bacteriophage type (9–11). NEP promoters are similar to mitochondrial promoters. Most NEP promoters share with most mitochondrial promoters a conserved YRTA motif located upstream of the transcription initiation site. In addition, there exist non-consensus-type mitochondrial and plastid NEP promoters that lack the YRTA sequence. If present, this motif is crucial for promoter recognition (10,11). The nuclear genome of *Arabidopsis thaliana* contains three genes for phage-type RNA polymerases coding for a mitochondrial enzyme (RpoTm), a chloroplast enzyme (RpoTp) and an RNA polymerase (RpoTmp) likely targeted to both mitochondria and plastids (12,13). Thus, two distinct phage-type enzymes may contribute to NEP activity. *Nicotiana tabacum*, an allotetraploid species, possesses six genes for organellar phage-type RNA polymerases [two sets of RpoTm, RpoTp and RpoTmp, (14)]. *RpoTp* and *RpoTmp* are proposed to have evolved from the gene for

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the mitochondrial RNA polymerase by duplication events (13). The plastid RNA polymerase RpoTp from *Arabidopsis* has recently be shown to recognize mitochondrial promoters nearly as efficient as the mitochondrial RNA polymerase RpoTm in *in vitro* assays, while RpoTmp did not show a preference for any plastid or mitochondrial promoter over random initiation sites (15). These data may suggest that RpoTp has kept the intrinsic capability of mitochondrial promoter recognition during evolution towards a chloroplast transcriptase and raises the question, if mitochondrial promoters would be able to drive transcription in the chloroplast also *in vivo*, where transcription factor(s) are thought to support RpoTp and RpoTmp in promoter recognition (15).

To study the activity of a plant mitochondrial promoter in plastids, we chose one of the best-characterized mitochondrial promoters, the *atpA* promoter from *Oenothera berteriana* (16). *In vitro* capping experiments had shown that the identified 5′ end of the *atpA* mRNA represents a genuine transcription initiation site (16). It is located immediately downstream of a conserved sequence block containing the crucial YRTA motif. We report here on the faithful recognition of the *Oenothera atpA* promoter by the *Arabidopsis* RNA polymerases RpoTm and RpoTp in *in vitro* transcription assays. Moreover, we show the accurate functioning of the mitochondrial promoter in plastids by demonstrating *atpA* promoter-driven transcription of a reporter gene in transgenic tobacco chloroplasts.

MATERIALS AND METHODS

Plant material and growth conditions

Sterile tobacco plants (*N. tabacum* cv. Petit Havana) were grown on agar-solidified MS medium containing 30 g/l sucrose (17). Homoplasmic transplastomic lines were rooted and propagated on the same medium. To perform crosses and obtain seeds, transplastomic plants were grown to maturity under standard glasshouse conditions. Seedling phenotypes were analysed by germination of seeds on MS medium containing spectinomycin (500 mg/l) or different concentrations of kanamycin. Kanamycin exposure experiments with leaf explants were performed on RMOP regeneration medium (18) containing different concentrations of kanamycin.

Construction of plastid transformation vectors

The plastid transformation vectors constructed in this study are based on the previously described vectors pRB94 and pRB95 (19). A chimeric *nptII* gene with mitochondrial expression signals was constructed by fusion of the *nptII* coding region to the PCR amplified mitochondrial *atpA* promoter and the 3′ untranslated region (UTR) from the mitochondrial *atp9* gene. The *atpA* (*atp1*) promoter from *O. berteriana* (16) was amplified with primers PPatpA-5′ (5′-TTTTGAGCTCTTTCCAATCCGGTTCCAAG-3′) and PPatpA-3′ (5′-TTTTCCATGGTTCAATTCGATTTTG-3′). With the primer sequences, a 5′ SacI site and a 3′ NcoI site were introduced (restrictions sites underlined in primer sequences) and used for subsequent cloning of the promoter fragment (Figure 2). The *atp9* terminator region from pea [*Pisum sativum*, (20)] was amplified with primer pair PTatp9-5′ (5′-TTTTTCTAGATTTATCCTTTTGAAAGGTG-3′) and PTatp9-3′ (5′-TTTTAAGCTTCAAAGCAAGCAAACTAACG-3′) and cloned as XbaI/HindIII fragment (restriction sites underlined in primer sequences; Figure 2). Correctness of PCR amplification and cloning of the expression elements were verified by control sequencing. The chimeric *nptII* cassette was integrated as SacI/HindIII fragment into plasmid pRB94 (19) generating vector pSR7. Integration of the same SacI/HindIII fragment into plasmid pRB95 (19) produced transformation vector pSR8 (Figure 2).

Plastid transformation and selection of homoplasmic transplastomic tobacco lines

Young leaves from sterile tobacco plants were bombarded with plasmid-coated 0.6 µm gold particles using a biolistic gun (PDS1000He; BioRad). Primary spectinomycin-resistant lines were selected on RMOP regeneration medium containing 500 mg/l spectinomycin (18,21). Spontaneous spectinomycin-resistant plants were eliminated by double selection on medium containing spectinomycin and streptomycin [500 mg/l each, (21,22)]. For each construct, several independent transplastomic lines were subjected to four additional rounds of regeneration on RMOP/spectinomycin to enrich the transplastome and select for homoplasmic tissue.

Isolation of nucleic acids and hybridization procedures

Total plant DNA was isolated by a rapid cetyltrimethylammoniumbromide (CTAB)-based miniprep procedure (23). DNA samples were digested with restriction enzymes, separated on 0.8% agarose gels and blotted onto Hybond N nylon membranes (Amersham). For hybridization, $\alpha[^{32}P]$dCTP-labeled probes were generated by random priming (Multiprime DNA labelling kit, Amersham). A StyI/PstI restriction fragment (Figure 2) was used as probe for the restriction fragment length polymorphism (RFLP) analyses. An NcoI/XbaI fragment covering the entire *nptII* coding region was used to detect *nptII* transcripts by northern blotting. Hybridizations were carried out at 65°C in Rapid Hybridization Buffer (Amersham) following the manufacturer's protocol.

5′-end mapping of *in vivo* and *in vitro*-synthesized RNAs

5′ termini of *in vivo* synthesized transcripts were determined employing a 5′-RACE technique combined with tobacco acid pyrophosphatase (TAP) treatment as described by Kühn *et al.* (24) with the following modifications: The gene-specific primer used for reverse transcription was Pa (5′-CTGTTGTGCCCAGTCATAG-3′). The products of reverse transcription (RT) were amplified in a first PCR step by using 4 µl of the RT reaction, 5 pmol of both the adapter-specific forward primer P1a (5′-CGAATTCCTGTAGAACGAACACTAGAAG-3′) and the gene-specific reverse primer Pb (5′-ATAGTTTGTGGAGAATTTCGCTTCC-3′), 200 µM of each dNTP and 0.5 U of *Taq* DNA polymerase (Qiagen) in 25 µl of the appropriate buffer. Amplification conditions comprised an initial denaturation step at 94°C for 1 min followed by 35 cycles of 95°C for 20 s, 58°C for 20 s and 72°C for 2 min and a final extension step at 72°C for 10 min. A 1 µl aliquot of the first PCR reaction was used as template for subsequent nested PCRs set up essentially as the first PCR in a volume of 50 µl with 10 pmol of both the gene-specific primer Pc (5′-CTGTGTTAAGCATAGGGCCTAACTAGC′3′) and the adapter-specific primer P1a.

To determine the 5′ ends of *in vitro*-synthesized transcripts, transcription assays and 5′ end mapping were carried out as described previously (15) with the following minor modifications: the RNA adapter sequence was changed to 5′-GUGAUCCAACCGACGCGACAAGCUAAUGCAAGANNN-3′. Reverse primers P2hisa (5′-CACATCGCCTGAAAGACT-3′) and P3hisa (5′-GGATGATGGTGATGATGGTGG-3′) annealing to the *hisa* attenuator sequence were used for cDNA synthesis and PCR, respectively. Primer RUMSH1 (5′-TGATCCAACCGACGCGAC-3′) annealing to the 5′ adapter sequence served as forward primer in the PCR reaction. For positions of primers P2hisa and P3hisa, see Figure 1A.

Chloroplast isolation and run-on transcription

Chloroplast isolation was performed according to Gruissem *et al.* (25). Wild-type and mutant plants were grown in soil under standard conditions. Leaves (6 g) from 4-week-old plants were homogenized in 150 ml isolation buffer (25 mM HEPES–KOH, pH 7.6, 350 mM sorbitol, 0.4 mM Na-isoascorbate, 2 mM EDTA) in a Waring blender. Intact plastids were isolated by centrifugation (6500*g*, 4°C, 20 min) in 40%/80% discontinuous Percoll gradients and collected at the gradient interface. Subsequently, the chloroplasts were resuspended in 200 µl isolation buffer. The chloroplast number was determined by counting in a hemocytometer and adjusted to 5×10^7. All procedures were performed at 4°C. Run-on transcription was carried out as described by Mullet and Klein (26) using 5×10^7 disrupted plastids in 100 µl reaction buffer containing 50 mM Tris–HCl pH 8.0, 10 mM MgCl$_2$, 0.2 mM CTP, GTP, ATP, 0.01 mM UTP, 50 µCi of α-^{32}P-UTP (Amersham), 40 U of RNase inhibitor (Fermentas) and 10 mM β-mercaptoethanol. After a 15 min incubation at 25°C the reaction was stopped by adding an equal volume of stop solution (50 mM Tris–HCl, pH 8.0, 25 mM EDTA, 5% sarcosyl).

Radioactive run-on transcripts were purified by phenol extraction according to Gaudino and Pikaard (27) and hybridized with 1 µg of non-radiolabeled *rpoB*, *clpP*, *psbA* and *nptII* gene-specific fragments dot blotted onto nylon Hybond-N$^+$ membranes (Amersham). The gene fragments were amplified from plastid or cloned plasmid DNAs by PCR using the following primers: rpoB-fw (5′-GCTGCTTGTCGTTCCAACC-3′) and rpoB-rev (5′-GGATTGGCTCTGGTTCGTTTAG-3′), clpP-fw (5′-GGCCAAGAGGTTGATAGCGAGATTTC-3′) and clpP-rev (5′-GCTAGACGTTTGGTAATTTCTCCTCCGAC-3′), psbA-fw (5′-ACTTGGCGTAGCTTGTTACATGGGTC-3′) and psbA-rev (5′-ACTGAATAGGGAGCCGCCGAATA-3′). The hybridization results were analysed with a PhosphorImager (Molecular Imager FX, Bio-Rad).

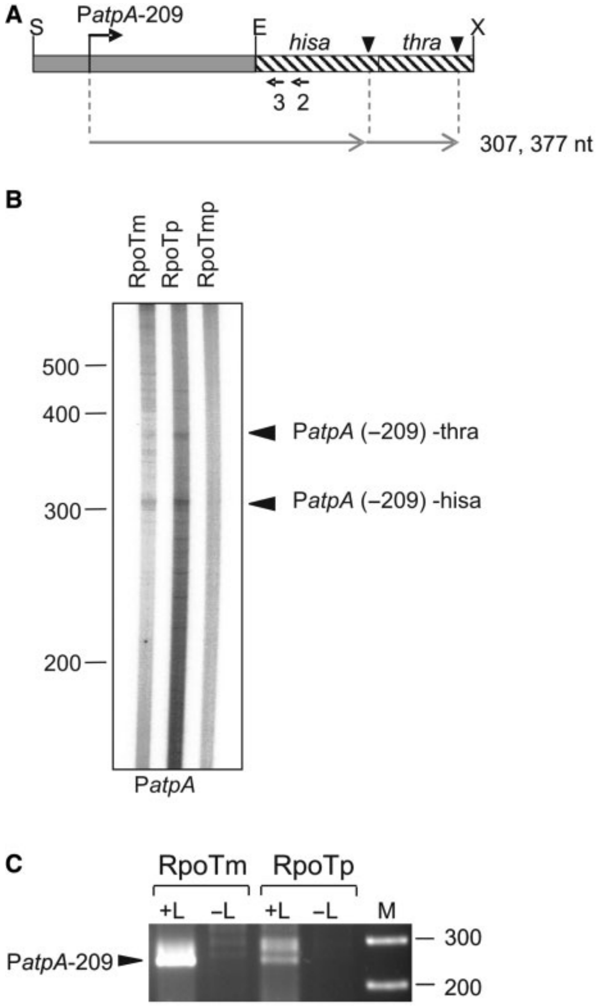

Figure 1. *In vitro* transcription from the mitochondrial *atpA* promoter (P*atpA*-209) by recombinant AtRpoTm, AtRpoTp and AtRpoTmp. (**A**) Schematic map of the plasmid used as template for *in vitro* transcription assays. Transcripts expected from initiation at P*atpA*-209 (bent arrow) and termination at *hisa* or *thra* (filled triangles) are indicated by the grey horizontal arrows below the gene map, the respective RNA lengths are indicated. Location and orientation of primers P2*hisa* (2) and P3*hisa* (3) employed for transcript 5′ end mapping are also shown. Restriction sites are abbreviated as follows: S, SacI; E, EcoRI; X, XhoI. (**B**) *In vitro* transcription products obtained with the three *Arabidopsis* phage-type organellar RNA polymerases. Recombinant enzymes AtRpoTm, AtRpoTp and AtRpoTmp were assayed for promoter-specific transcription from the supercoiled plasmid pKL23-*atpA*. Labeled RNA products were separated in a 5% sequencing gel. Fragment lengths of the RNA size marker are given in nucleotides at the left. Transcripts resulting from initiation at P*atpA*-209 followed by termination at *hisa* or *thra* are indicated by arrowheads. (**C**) Mapping of mRNA 5′ ends. 5′-RACE was performed on RNAs synthesized *in vitro* from pKL23-*atpA* by AtRpoTm and AtRpoTp. Transcripts 5′ ligated to an RNA linker (lane +L) and, as a control, non-ligated transcripts (–L) were subjected to RT-PCR. PCR products were separated in an agarose gel, fragment sizes of the DNA molecular weight marker are given in base pairs. Signals that correspond in size to transcript 5′ ends arising from P*atpA*-209 are indicated by the arrowhead.

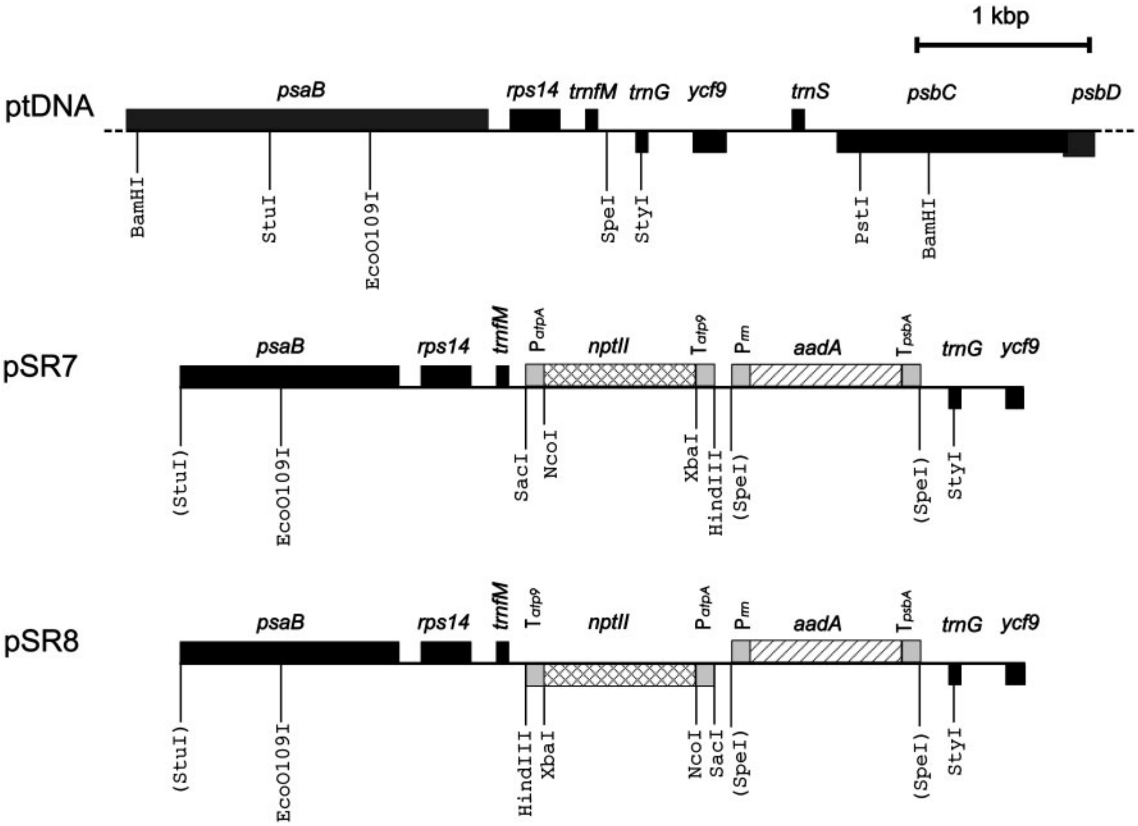

Figure 2. Construction of plastid transformation vectors for the expression of a chimeric *nptII* gene under the control of a mitochondrial promoter. Physical maps of the targeting region in the tobacco plastid genome and the two plastid transformation vectors, pSR7 and pSR8, are shown. Genes above the lines are transcribed from the left to the right, genes below the lines are transcribed in the opposite direction. Restriction sites used for cloning, RFLP analysis and/or generation of hybridization probes are indicated. Sites lost due to ligation to heterologous ends are shown in parentheses. Note that the selectable marker gene for chloroplast transformation, *aadA*, is under the control of plastid-specific expression signals, whereas the kanamycin resistance gene *nptII* is driven by mitochondrial expression signals. P*rrn*, rRNA operon-derived plastid promoter (21); T*psbA*, 3′ UTR derived from the plastid *psbA* gene; P*atpA*, mitochondrial *atpA* promoter; T*atp9*, 3′ UTR from the mitochondrial *atp9* gene.

In vitro transcription

Expression of recombinant RNA polymerases from *Arabidopsis* (AtRpoTm, AtRpoTp, AtRpoTmp) in *Escherichia coli*, their purification, *in vitro* transcription assays as well as the analysis of transcription products by polyacrylamide gel electrophoresis (PAGE) were performed as described previously (15). For preparation of the *in vitro* transcription template, the *atpA* promoter fragment was PCR-amplified from pSR8 plasmid DNA with the following primer pair: Ps-P*atpA*-fw(SacI) 5′-GCAGAGCTCAGCTCTTTCCAATCCGGTTC-3′/Ps-P*atpA*-rev(EcoRI) 5′-GCAGAATTCTCTCGCCCAGAGAAAAGAAA-3′ (restriction sites underlined). The PCR product was digested with SalI and EcoRI and ligated into the SalI/EcoRI-cleaved plasmid pKL23 (28) upstream of the terminator sequences. The cloned template was purified from *E. coli* using the QIAGEN Plasmid Midi Kit.

Protein extraction and immunoblot analyses

Total soluble protein (TSP) was extracted from leaf samples homogenized in a buffer containing 50 mM HEPES–KOH (pH 7.5), 10 mM KAc, 5 mM MgAc, 1 mM EDTA, 1 mM DTT and 1 mM Pefabloc followed by centrifugation to remove insoluble material. Samples representing 10 µg of extracted proteins were separated by electrophoresis in 15% SDS–polyacrylamide gels and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Amersham). The NptII protein was detected with a specific anti-NptII antibody generated in rabbits (LINARIS GmbH, Wertheim-Bettingen, Germany). Immunobiochemical detection was done using the ECL Plus detection system (Amersham) according to the instructions of the manufacturer.

RESULTS

Recognition of the mitochondrial *atpA* promoter by plastid and mitochondrial phage-type RNA polymerases *in vitro*

In order to test whether the *Arabidopsis* RNA polymerases RpoTm, RpoTp and RpoTmp possess the intrinsic capacity of recognizing the *atpA* promoter from *Oenothera* mitochondria, we conducted *in vitro* transcription assays using purified recombinant RNA polymerases. We will subsequently refer to the promoter as 'P*atpA*-209' according to the transcription initiation site of the *atpA* gene which is located 209 nucleotides upstream of the first translated nucleotide (+1) of the *atpA* mRNA. The DNA template was constructed by inserting the *atpA* promoter region into plasmid pKL23 (28) upstream of the two bacterial ρ-independent terminator sequences *hisa* (29) and *thra* (30). When supercoiled pKL23-*atpA* is provided as template, any transcription initiated at the introduced promoter will be terminated specifically at *hisa* or *thra*, thereby generating RNA products of distinct lengths (Figure 1A). Figure 1B shows the gel electrophoretic analysis of RNAs synthesized *in vitro* from supercoiled pKL23-*atpA* by recombinant AtRpoTs. Transcription by AtRpoTm and AtRpoTp yielded two major discrete RNA products of ~310 and 380 nt (Figure 1B; black arrowheads), as expected if transcription initiated at P*atpA*-209. In contrast, no specifically initiated transcripts were produced by AtRpoTmp. In order to confirm correct initiation of RNA polymerization at position −209, we determined the 5′ termini of the discrete transcripts synthesized by AtRpoTm and AtRpoTp *in vitro* using 5′-RACE (Figure 1C). This technique comprises reverse transcription of 5′ linker-ligated RNAs, followed by PCR amplification of 5′ ends using a linker-specific forward primer and a transcript-specific reverse primer (Figure 1C, lanes +L). As a control, *in vitro* transcription products not ligated to the 5′ linker were subjected to RT-PCR (Figure 1C, lanes −L), thereby allowing to distinguish RNA-derived PCR products from signals resulting from non-specific amplification of residual contaminating DNA template molecules.

Sequencing of the cloned PCR products revealed that AtRpoTm perfectly recognized the *Oenothera* P*atpA*-209 promoter. All transcripts were found to have initiated exactly at the −209 site reported to serve for transcription initiation in *O. berteriana* mitochondria (16). In the case of the AtRpoTp-derived transcripts, about one-third of the determined 5′ termini corresponded exactly to the −209 initiation site, whereas the 5′ ends of most of the remaining transcripts mapped to positions one to five nucleotides upstream or downstream of the −209 start site, or to an AT-rich region ~30 nt upstream of the −209 site (Figure 1C, data not shown). Thus, AtRpoTm and AtRpoTp correctly recognized the mitochondrial promoter and faithfully initiated transcription from P*atpA*-209 *in vitro*, although AtRpoTp did so less precisely than AtRpoTm.

Integration of a transgene with mitochondrial expression signals into the tobacco plastid genome

Having obtained *in vitro* evidence for transcription from the mitochondrial *atpA* promoter by the plastid phage-type polymerase RpoTp, we were interested in providing direct *in vivo* proof for the recognition of the *atpA* promoter by the phage-type NEP. We, therefore, designed a chimeric reporter gene construct, in which we fused the coding region of a kanamycin resistance gene (*nptII*) to the mitochondrial *atpA* promoter (Figure 2). *nptII* was chosen as a reporter gene, because (i) its expression in plastids can be easily assayed *in planta* by the level of kanamycin resistance conferred by the NptII protein (31), and (ii) highly specific antibodies allow the sensitive detection and quantitation of NptII (31,32). To generate a true mitochondrial-type gene, we used, in addition to a mitochondrial promoter, also a mitochondrial 3′ UTR which was taken from the *atp9* gene (20,33). 3′ UTRs in both plastids and mitochondria usually fold into stable stem-loop-type RNA secondary structures, which, rather than terminating transcription, trigger 3′ end processing and confer RNA stability (33–35). As stable mRNA 3′ end formation is mediated by RNA secondary structure rather than primary sequence, the utilization of a mitochondrial 3′ UTR in plastids is not expected to have a significant effect on RNA stability.

With this chimeric mitochondrial-type *nptII* cassette, two plastid transformation vectors were constructed. In vector pSR7, the *nptII* has the same transcriptional orientation as the upstream *trnfM* gene and the downstream selectable marker gene *aadA* (conferring resistance to the aminoglycoside antibiotics spectinomycin and streptomycin; Figure 2). As with this construct, *nptII* mRNA accumulation could, at least in part, come from co-transcription with *trnfM* (due to read-through caused by incomplete transcription termination; (36), we also generated a plastid transformation vector with the opposite orientation of the *nptII* cassette (pSR8; Figure 2), thereby eliminating the possibility of read-through transcription.

The transformation vectors were introduced into plastids on the surface of 0.6 µm gold particles followed by selection for spectinomycin resistance conferred by the *aadA* marker gene (21). For both constructs, numerous antibiotic-resistant lines were obtained and successful chloroplast transformation was tentatively confirmed by double resistance tests on medium containing both spectinomycin and streptomycin, a test which eliminates spontaneous spectinomycin-resistant lines (18,21,22). Putative plastid transformants were passed through additional rounds of selection and regeneration to enrich the transgenic plastid genome and eliminate residual wild-type genome copies (21,22). After the third-regeneration round, RFLP analyses were conducted to verify successful plastid transformation, confirm correct transgene integration into the intergenic spacer region between the *trnfM* and *trnG* genes (Figure 2) and test for the presence of a homogeneous population of transgenic plastid genomes (homoplasmy). When probed with a radiolabeled fragment suitable to distinguish between wild-type and transformed genomes, the RFLP analyses revealed the expected size difference between the two genome types as caused by integration of the two transgenes (*nptII* and *aadA*) into the *trnfM*/*trnG* spacer of the tobacco plastid genome (Figure 3A, data not shown).

In addition to a strong band for the expected restriction fragment, RFLP analysis of both the pSR7 and pSR8-derived transplastomic tobacco lines also showed a faint hybridization signal that corresponds in size to the wild-type fragment (Figure 3A). Persistence of a wild-type-like hybridization signal even after multiple rounds of selection and regeneration is often seen in plastid transformation experiments and normally, does not come

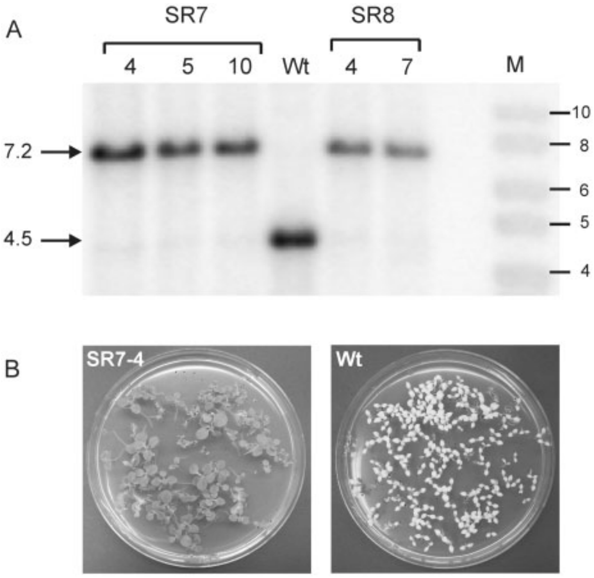

Figure 3. Molecular and genetic analysis of plastid transformants. (**A**) RFLP analysis of transplastomic lines produced with vectors pSR7 and pSR8. Total cellular DNA was digested with BamHI and hybridized to a radiolabeled probe (StyI/PstI fragment; see Figure 2) detecting the region of the plastid genome that flanks the transgene insertion site. Fragment sizes for the wild type (Wt) and the transplastomic lines are indicated in kb. M: molecular weight marker. Note that a very faint wild-type-like band is seen in all transgenic lines. This weak hybridization signal comes from promiscuous DNA that presumably resides in the nuclear genome (see text for details). (**B**) Example of a seed assay to confirm homoplasmy of transplastomic lines. Due to maternal inheritance of plastids in tobacco, homoplasmic transplastomic lines produce progeny that is homogeneously resistant to spectinomycin. Lack of phenotypic segregation in the T_1 generation, therefore, demonstrates homoplasmy. Wt: wild-type control displaying spectinomycin sensitivity.

from true heteroplasmy of the plastid transformants. Instead, it is caused by the presence of so-called 'promiscuous' plastid DNA in one of the other two genomes of the plant cell, in the nucleus or the mitochondrion. During evolution, large fragments of chloroplast DNA have integrated into the nuclear and mitochondrial genomes (3) and this non-functional promiscuous DNA can produce wild-type-like bands in Southern blot analyses of otherwise homoplasmic transplastomic lines (36,37). Seed assays provide a simple and reliable method to distinguish between promiscuous DNA and heteroplasmy (22). When we tested our pSR7 and pSR8-derived transplastomic lines for segregation of the spectinomycin resistance in the T1 generation, the progeny turned out to be homogeneously resistant to the antibiotic, demonstrating homoplasmy of the transgenic plastid genome (Figure 3B, data not shown) and confirming our earlier finding that tobacco harbors promiscuous DNA homologous to the targeting region in the plastid genome used in this study (38,39).

Expression of the mitochondrial-type *nptII* transgene in plastids

We next wanted to test whether the mitochondrial *nptII* transgene is expressed from the transgenic plastid genome. First, we determined transcript patterns and RNA accumulation in the SR7 and SR8 transplastomic lines by northern blot experiments. RNA accumulation was compared with two transgenic lines that strongly express *nptII* gene versions under different expression signals: a nuclear-transgenic line containing an *nptII* transgene under the control of CaMV35S promoter and terminator (39,40) and a chloroplast-transformed line in which the *nptII* was driven by the strongest available plastid promoter, the ribosomal RNA operon promoter P*rrn* (21,31). The P*rrn* promoter contains recognition elements for both PEP and NEP (41) with the PEP promoter making the predominant contribution to RNA accumulation in green tissue (42). As expected, *nptII* mRNA accumulation from the phage-type mitochondrial promoter was much lower than from the extremely strong P*rrn* and CaMV35S promoters (Figure 4A). Nonetheless, accumulation of an *nptII* transcript corresponding in size to the ~1 kb monocistronic mRNA in the control transformants was clearly detectable in both SR7 and SR8 transplastomic lines. The transcript patterns of the SR7 and SR8 transplastomic lines differed in that the SR7 lines showed an additional major RNA species of ~2.3 kb corresponding in size to a dicistronic *nptII–aadA* read-through transcript. Read-through transcription due to incomplete transcription termination is quite common in plastids (38,43). In fact, a read-through transcript with the downstream *aadA* has been observed before for other transgenes inserted into the plastid transformation vector used to construct pSR7 [vector pRB94, (19,38)]. Interestingly, accumulation of this additional co-transcript did not occur at the expense of the accumulation of the monocistronic *nptII* mRNA (Figure 4A) and consequently, SR7 lines contain about twice as much *nptII* RNA as SR8 lines.

Next, we analysed accumulation of the *nptII* gene product, the neomycin phosphotransferase protein, NptII. Using a specific anti-NptII antibody, protein accumulation in SR7 and SR8 lines was compared with that in the two control transformants. Correlating with the much lower mRNA accumulation levels, also NptII protein accumulation was much lower in the SR7 and SR8 transplastomic lines than in the 35S:*nptII* and P*rrn*:*nptII* control transformants (Figure 4B). As NptII protein levels in the SR7 and SR8 transformants were on the borderline of detectability, we conducted kanamycin resistance assays to ultimately confirm expression of the mitochondrial-type *nptII* gene in plastids at the protein level and, simultaneously, test for the accumulation of active NptII enzyme. Resistance tests were performed both with leaf explants exposed to regeneration media containing different concentrations of kanamycin (Figure 5A, data not shown) and with seeds germinated in the presence of the antibiotic (Figure 5B). Both sets of tests revealed a significant resistance to kanamycin in the SR7 and SR8 transplastomic lines (Figure 5), confirming expression of the *nptII* from the mitochondrial expression cassette at the protein level and moreover, demonstrating that the NptII protein produced from the mitochondrial-type gene in plastids is enzymatically active.

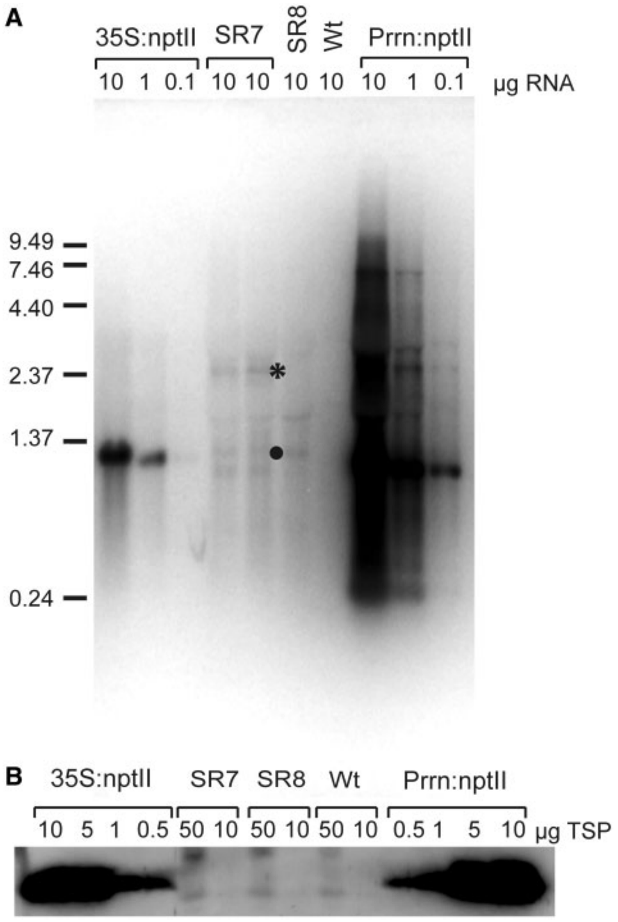

Figure 4. Expression of the mitochondrial-type *nptII* transgene in tobacco plastids. (**A**) Analysis of *nptII* transcript patterns and mRNA accumulation for two independently generated pSR7-derived transformants and a pSR8-derived transformant. For comparison, a dilution series for a nuclear transformant (35S:*nptII*) is shown, in which the *nptII* is under the control of the strong constitutive CaMV 35S promoter. A dilution series for a chloroplast transformant (P*rrn*:*nptII*), in which *nptII* is driven by the strongest known plastid promoter, the ribosomal RNA operon promoter P*rrn*, is also shown. The amount of mRNA loaded in each lane is indicated, the sizes of the bands of the molecular weight marker are given at the left. Note that the SR7 lines show an additional band at ~2.3 kb which corresponds in size to the *nptII–aadA* read-through transcript (marked by an asterisk; see Figure 2). Additional, minor RNA species were not further characterized. (**B**) Analysis of NptII protein accumulation using an anti-NptII antibody. The amounts of total soluble protein (TSP) loaded in each lane are indicated. Compared to the strongly expressing nuclear and plastid *nptII* gene versions, the sensitivity of the anti-NptII antibody is insufficient to clearly detect the protein expressed from the mitochondrial-type *nptII* in plastids (see Figure 5). By and large, protein amounts correlate with mRNA levels and the undetectably low NptII levels obtained with the mitochondrial-type *nptII* reflect the much lower mRNA accumulation levels in the SR7 and SR8 lines.

Interestingly, in both assays, kanamycin resistance was significantly stronger in the SR7 transplastomic lines than in the SR8 lines (Figure 5). This is most readily explained by the higher *nptII* mRNA levels in the SR7 lines due to accumulation of additional *nptII–aadA* read-through transcripts.

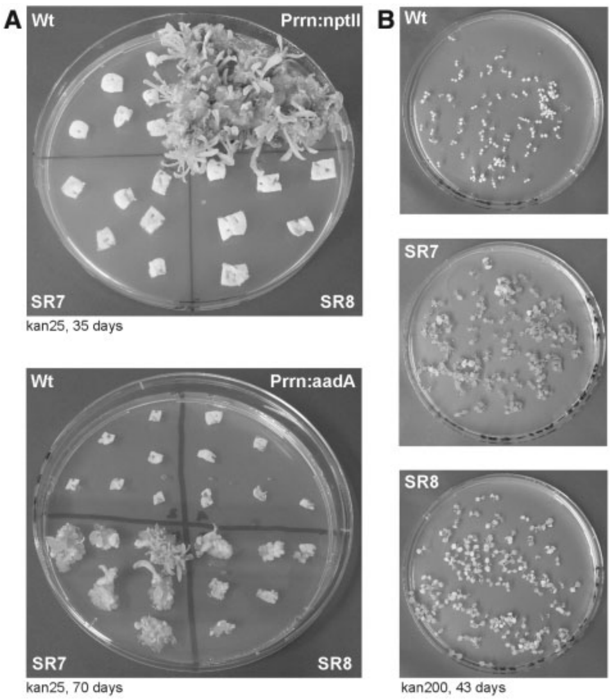

Figure 5. Confirmation of the expression in plastids of functional enzyme from the mitochondrial-type *nptII* by phenotypic assays. (**A**) Determination of kanamycin resistance by exposure of leaf explants to kanamycin-containing plant regeneration medium. After 35 days, only regeneration from leaf pieces of the control plastid transformant (line P*rrn*:*nptII*) containing an *nptII* under the control of the strongest available plastid promoter is seen (upper picture). However, after 70 days on kanamycin-containing medium, regeneration of SR7 and SR8 lines is obtained, whereas explants from the wild-type control (Wt) and a control plastid transformant harboring just the selectable marker gene *aadA* (P*rrn*:*aadA*) are fully bleached out due to their sensitivity to the antibiotic. (**B**) Analysis of kanamycin resistance by seed assays. Note that in both (**A**) and (**B**), the higher level of *nptII* expression in the SR7 lines (due to the presence of additional read-through transcripts; see Figure 4) correlated with stronger phenotypic resistance to kanamycin. Kanamycin (kan) concentrations are given in µg/ml.

Analysis of transcriptional activity and mapping of transcription start sites

The data described above imply transcription of the plastome-integrated *nptII*-cassette under control of the mitochondrial *atpA* promoter. However, the observed monocistronic *nptII* transcript (Figure 4A) could come from transcription initiation at the P*atpA*-209 promoter or, alternatively, from processing of larger precursor RNAs whose transcription initiates at an upstream plastid promoter. To distinguish between these possibilities, we decided to map the 5′ ends of the *in vivo* synthesized *nptII* transcripts. To precisely determine the initiation sites of the *nptII* transcripts, we used a modified 5′-RACE technique suitable to selectively detect 5′ ends of primary transcripts (24,44). The primary 5′ ends of organellar transcripts carry triphosphates, whereas processed transcripts have monophosphates at their 5′ ends. RNA ligase can ligate an RNA linker only to the latter, because 5′ triphosphates do not serve as a substrate for the enzyme. Therefore, primary 5′ termini can be ligated

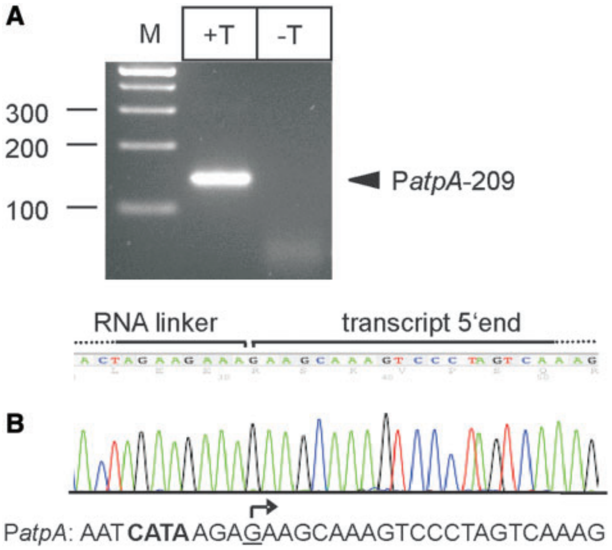

Figure 6. Mapping of 5′ ends of *in vivo* synthesized *nptII* transcripts. (**A**) 5′-RACE analysis of *nptII* transcripts. Amplified products were separated by agarose gel electrophoresis, fragment sizes of the DNA molecular weight marker are given in base pairs. TAP-specific products (lane +T) that correspond to the 5′ ends of primary transcript are indicated by an arrow and labeled with the position in respect to the translation initiation site (+1). 5′-RACE of RNA in TAP buffer without TAP treatment was performed as a control (lanes –T). (**B**) Chromatogram displaying the sequence at the ligation site of a typical cloned 5′-RACE product. Below the chromatogram, the sequence of the *atpA* promoter region with the core promoter motif marked in boldface letters is shown. The transcription initiation site is underlined and marked by the bent arrow.

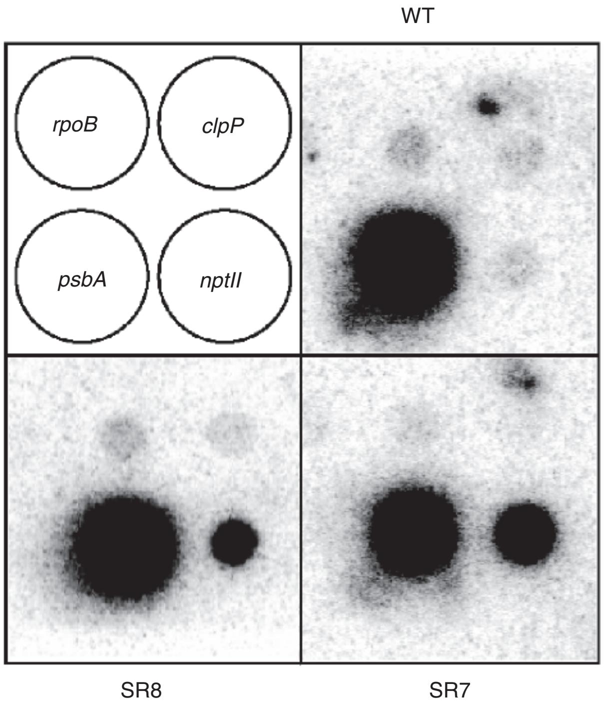

Figure 7. Detection of newly synthesized *nptII* transcripts in isolated transgenic plastids. The spotting scheme and dot-blot autoradiograms of chloroplast run-on transcription experiments are shown. Chloroplasts were isolated from 4-week-old transplastomic (SR7/SR8) or wild-type (WT) plants. Transcriptional activity from the mitochondrial P*atpA* promoter driving the chimeric *nptII* gene in plastids is compared with that of representative resident plastid genes (*rpoB*, *clpP*, *psbA*).

only after enzymatic removal of the 5′ pyrophosphate, a reaction catalysed, for example, by TAP. As a consequence, 5′-RACE will yield products from TAP-treated RNA for both primary and processed transcripts, whereas without exposure to TAP, products resulting from primary transcript termini will be significantly reduced or absent. In this way, comparison of 5′-RACE products obtained from TAP-treated and untreated RNA (lanes +T and −T in Figure 6) identified the 5′ ends of the primary *nptII* transcripts synthesized in transplastomic plants. Interestingly, the 5′ termini corresponded exactly to the 5’ end of the native *atpA* transcript initiated at P*atpA*-209 in mitochondria [(16), Figure 6]. Remarkably, in contrast to the situation in our *in vitro* assays, all transcripts were found to have the correct 5′ end at position −209.

To compare the strength of the *atpA* promoter with plastid NEP promoters and a strong PEP promoter, we carried out plastid run-on assays using isolated chloroplasts from the SR7 and SR8 lines (Figure 7). α-^{32}P-UTP labeled nascent RNAs were hybridized to filters containing dot-blotted sequences of the *nptII* gene and of the resident chloroplast genes *rpoB* (transcribed by NEP), *clpP* [transcribed from three NEP promoters and one PEP promoter, (11)] and *psbA* (transcribed by PEP). This hybridization experiment confirmed the active transcription of the *nptII* gene in the transplastomic lines SR7 and SR8. As expected, run-on transcripts from wild-type chloroplasts used as control did not hybridize to the *nptII* probe on the filter. The P*atpA*-209 was weaker than the strong *psbA*-PEP promoter confirming the earlier finding that, in green tissues, PEP transcriptional activity is much stronger than NEP activity. Remarkably, the hybridization signals for the *nptII* transcript were distinctly stronger than the signals for the resident chloroplast genes *rpoB* and *clpP*, indicating that the mitochondrial promoter was more efficient in driving plastid transcription than the investigated chloroplast *rpoB and clpP* NEP promoters.

DISCUSSION

In this work, we have shown that a mitochondrial promoter is faithfully recognized by the nuclear-encoded plastid RNA polymerase both *in vitro* and *in vivo*. Transcription from the mitochondrial *atpA* promoter initiated at the identical site in mitochondria, in transgenic chloroplasts and in an *in vitro* transcription system using purified organellar RNA polymerases. It is well established that both prokaryotic and eukaryotic RNA polymerases require transcription factors for promoter recognition. In contrast, promoter recognition by the bacteriophage T7 RNA polymerase is an intrinsic property of this single-polypeptide enzyme (45). This remarkable feature may have been retained during the evolution of organellar phage-type RNA polymerases from an ancestral bacteriophage enzyme (15,46). The yeast mitochondrial RNA polymerase (47) and the *Arabidopsis* RNA polymerases RpoTm and RpoTp (15) were shown to accurately recognize promoters *in vitro* without support from additional proteins. However, *in vivo*, effective transcription by the yeast enzyme is known to require transcription factors (47). A similar situation has been suggested for transcription in *Arabidopsis* mitochondria and chloroplasts, since RpoTm and RpoTp were not able to recognize *in vitro* all of the promoters they utilize *in vivo*, and RpoTmp did not even efficiently recognize any promoter *in vitro* (15). In the present investigation, we also observed no specific recognition of P*atpA*-209 by RpoTmp in *in vitro* assays. In contrast, *Arabidopsis* RpoTm, the mitochondrial RNA polymerase, precisely initiated transcription of *nptII* from the *Oenothera* mitochondrial promoter *in vitro*. Interestingly, also the plastid-targeted enzyme RpoTp was evidently able to recognize P*atpA*-209 as promoter, but initiated *in vitro* only about one-third of the transcripts correctly at nucleotide −209, the initiation site of the *atpA* promoter determined in mitochondria (16). Surprisingly, when integrated into the plastid genome, the mitochondrial *atpA* promoter served as a perfect chloroplast promoter: all *nptII* transcripts produced *in vivo* were found to have the correct 5′ end at position −209.

These findings raise interesting evolutionary considerations. First, the data from *in vitro* transcription assays support the idea that RpoTm and RpoTp have kept the intrinsic capability for promoter recognition during evolution from an ancestral phage polymerase, while RpoTmp has lost this property (15). The plastid enzyme RpoTp was less efficient than the mitochondrial RNA polymerase in correct initiation from the mitochondrial *atpA* promoter *in vitro*, possibly reflecting an evolutionary adaptation of RpoTp to plastid promoters. More detailed analyses of polymerase–promoter interactions will be required to test this hypothesis.

Second, faithful recognition of a mitochondrial promoter by the chloroplast transcriptional apparatus suggests that, despite evolutionary separation of the plastid and mitochondrial phage-type RNA polymerases [which presumably occurred early in the evolution of angiosperms, (11)], the mechanisms of promoter recognition have remained remarkably conserved in both organelles. Moreover, accurate recognition of P*atpA*-209 in transgenic chloroplasts contrasted with a less precise initiation by RpoTp *in vitro* and with the inability of RpoTmp to utilize this promoter in *in vitro* transcription assays. This suggests that one or several chloroplast transcription factor(s) may contribute to promoter recognition *in vivo*, possibly indicating that not only promoters and RNA polymerases, but also additional components of the plastid transcriptional apparatus, may be evolutionarily related to the corresponding factors in mitochondria. A reasonable explanation for such a striking conservation could be that the evolutionary leeway for transcription systems is strongly constrained by the requirement for co-evolution of promoters, RNA polymerases and their putative transcription factors.

Third, the faithful recognition of a mitochondrial promoter by plastid RNA polymerases *in vivo* (and, presumably, also of plastid promoters by mitochondrial RNA polymerases) may facilitate successful gene transfer events between organelles. It is well established that part of the plant mitochondrial tRNA genes are of chloroplast origin and were acquired by gene transfer from the plastid to the mitochondrial genome (3,48). It seems conceivable that the immediate functioning of transferred plastid tRNA genes in the mitochondrion allowed them to quickly replace their mitochondrial counterparts, which, in turn, prevented the rapid mutational decay that non-functional promiscuous DNA sequences are usually subject to. However, the structures of plastid and mitochondrial tRNA promoters are just beginning to emerge (49), and rigorous testing of this hypothesis will require a much better knowledge about the transcription of tRNA genes in both organelles.

By and large, low *nptII* mRNA accumulation levels in our transgenic lines correlated with low NptII protein accumulation (Figures 3–5) and the NptII protein produced from the mitochondrial expression cassette in plastids proved to be enzymatically active. This indicates that the plastid translational machinery can faithfully initiate protein biosynthesis from the mitochondrial 5′ UTR. Translation initiation signals in plant mitochondria are much less well defined than those in plastids, although some conserved sequence elements have been identified upstream of translational start codons in mitochondrial genes which could be involved in ribosome recruitment to the 5′ UTR (50,51). Plant mitochondrial 5′ UTRs usually lack Shine–Dalgarno-like sequence motifs that could act as ribosome-binding sites via complementary base pairing with the 3′ end of the 16S rRNA. In contrast, many plastid mRNAs show Shine–Dalgarno sequences which have been shown to be required for translation initiation (52,53). However, there are also plastid genes lacking an obvious Shine–Dalgarno sequence (54) and it is generally believed that mRNA-specific translational activator proteins recognize specific *cis*-elements in the 5′ UTRs of those mRNAs, recruit the ribosomes and, in this way, compensate for the lack of a Shine–Dalgarno-type ribosome-binding site (52,55,56). In view of these striking differences in the mechanisms of translation initiation between plastids and mitochondria it is interesting that the 5′ UTR from the mitochondrial *atpA* gene is capable of mediating translation of the *nptII* transgene in plastids. However, due to our limited knowledge about translation initiation signals in plant mitochondria, the molecular basis of this observation currently remains unexplained.

Finally, the functioning of mitochondrial expression signals in plastids, as demonstrated in this study, also adds mitochondrial promoters and 3′ UTRs to the toolbox for plastid genetic engineering. In this context it is interesting to note that, in our transplastomic experiments, the mitochondrial *atpA* promoter proved to be stronger than the resident chloroplast NEP promoters of *clpP* and *rpoB* (Figure 7). So far, chloroplast expression elements have been used nearly exclusively to drive transgene expression from the plastid genome (59).

One of the disadvantages of using plastid promoters and UTRs is that their use in transgene expression cassettes duplicates sequences in the chloroplast genome which can cause unwanted homologous recombination resulting in partial genome deletions or inversions (21,57,58). The attraction of using mitochondrial expression signals is that the introduction of homologous sequences can be avoided altogether, thereby greatly reducing the risk of recombination-induced genome instability. However, it should be noted that, while this represents a viable option for low- and medium-level transgene expression, the relatively moderate expression levels obtainable from phage-type promoters are unlikely to be sufficient to trigger high-level foreign protein accumulation as, for example, strived for in molecular farming (59). In fact, in all cases where very high transgene expression levels could be obtained in transplastomic plants, expression was driven by very strong PEP promoters (60–63). Nonetheless, for all those applications where moderate expression levels are desirable, such as, for metabolic pathway engineering (38,64,65), mitochondrial expression elements may provide a safer alternative to the use of endogenous plastid sequences. Finally, it should also be possible to improve plastid transgene expression at the translational level by modifying the mitochondrial 5′ UTR to contain a consensus Shine–Dalgarno sequence.

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