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 vitro assays suggesting the possibility that mitochondrial promoters might function as well in plastids. We have, therefore, generated transplastomic tobacco plants harboring 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
the mitochondrial RNA polymerase by duplication events (13). The plastid RNA polymerase RpoTp from Arabidopsis has recently been shown to recognize mitochondrial promoters nearly as efficiently 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). Kanamycin-resistant lines were selected on RMOP regeneration 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.

**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′-TTTTTCTAGATTTATCCCTTGGAAAGGTG-3′) and PPatpA-3′ (5′-TTTTTACCTG TTCAAATCGATTGTTTGG-3′). With the primer sequences, a 5′ SacI site and a 3′ NcoI site were introduced (restriction 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′-CTGTTGTGCCCAGTCATAG-3′) and PTatp9-3′ (5′-CTGTTGTGCCCAGTCATAG-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). A StyI/PstI restriction fragment (Figure 2) was amplified employing a 5′-32P-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′-CGAATTCTGGTAGAAGCAACTA GAAG-3′) and the gene-specific reverse primer Pb (5′-ATAGTTTTGTGGAGAATTTGCTTCC-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'-CTGTGTTAAGCATAGGCGCTAAGC/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'-GUGAUCCAAACCGACCGACAGCU AUGCAGAAGNN-3'. Reverse primers P2hisa (5'-CAC ATCGCCTGAAAGACT-3') and P3hisa (5'-GGATGA TGGTATGATGCCGG-3') annealing to the hisa attenuator sequence were used for cDNA synthesis and PCR, respectively. Primer RUMSH1 (5'-TGATCCAC CGACCCGAC-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 (6500g, 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 x 10^5. All procedures were performed at 4°C. Run-on transcription was carried out as described by Mullet and Klein (26) using 5 x 10^4 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-32P-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, ctpP, psbA and nptII gene-specific fragments dot blotted onto nylon Hybond-N+ membranes (Amersham). The gene fragments were amplified from plastid or supercoiled plasmid pKL23-arth. The fragments were 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 and the adapter-specific primer. The PCR products were separated on an agarose gel, fragment lengths of the DNA molecular weight marker are given in base pairs. Signals that correspond in size to transcript 5' ends are indicated by arrowheads. (B) Mapping of mRNA 5' ends. 5'-RACE was performed on RNAs synthesized in vitro from pKL23-rpoB by AtRpoTm and AtRpoTp. Transcripts recovered from the supercoiled plasmid pKL23-rpoB were separated by gel electrophoresis. Fragment lengths of the DNA molecular weight marker are given in nucleotides at the left. Transcripts resulting from initiation at P0 were followed by 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 P2hisa (2) and P3hisa (3) employed for transcript 5' end mapping are also shown. Restriction sites are abbreviated as follows: S, SacI; E, EcoRI; X, Xhol.

![Figure 1](image)

**Figure 1.** In vitro transcription from the mitochondrial rpoB promoter (PatpA-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 P0 by recombinant AtRpoTm, AtRpoTp and AtRpoTmp were assayed for promoter-specific transcription from the supercoiled plasmid pKL23-rpoB. The RNA lengths with 32P-UTP are indicated by arrowheads. (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-rpoB. 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 P0 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-rpoB 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 are indicated by the arrowhead.

AGCTTGTTACATGGGTC-3' and psbA-rev (5'-ACTG AATAGGGAGCCGCGCAATA-3'). The hybridization results were analysed with a PhosphorImager (Molecular Imager FX, Bio-Rad).
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-PatpA-fw(Sacl) 5'-GCAGAGCTCTAGCTTTTCTTCTACGCATCCGG GTTC-3'/Ps-PatpA-rev(EcoRI) 5'-GCAGAATTCTCTG CCCCAGAGAAAAGAAA-3' (restriction sites underlined). The PCR product was digested with SaII and EcoRI and ligated into the SaII/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 ‘PatpA-209’...
according to the transcription initiation site of the \textit{atpA} gene which is located 209 nucleotides upstream of the first translated nucleotide (+1) of the \textit{atpA} mRNA. The DNA template was constructed by inserting the \textit{atpA} promoter region into plasmid pKL23 (28) upstream of the two bacterial \textit{p}-independent terminator sequences \textit{hisa} (29) and \textit{thra} (30). When supercoiled pKL23-\textit{atpA} is provided as template, any transcription initiated at the introduced promoter will be terminated specifically at \textit{hisa} or \textit{thra}, thereby generating RNA products of distinct lengths (Figure 1A). Figure 1B shows the gel electrophoretic analysis of RNAs synthesized \textit{in vitro} from supercoiled pKL23-\textit{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 \textit{PatpA}-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 \textit{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, \textit{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 \textit{Oenothera PatpA}-209 promoter. All transcripts were found to have initiated exactly at the −209 site reported to serve for transcription initiation in \textit{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 \textit{PatpA}-209 \textit{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 \textit{in vitro} evidence for transcription from the mitochondrial \textit{atpA} promoter by the plastid phage-type polymerase RpoTp, we were interested in providing direct \textit{in vivo} proof for the recognition of the \textit{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 (\textit{nptII}) to the mitochondrial \textit{atpA} promoter (Figure 2). \textit{nptII} was chosen as a reporter gene, because (i) its expression in plastids can be easily assayed \textit{in planta} by the level of kanamycin resistance conferred by the NptIII 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 \textit{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 \textit{nptII} cassette, two plastid transformation vectors were constructed. In vector pSR7, the \textit{nptII} has the same transcriptional orientation as the upstream \textit{trnfM} gene and the downstream selectable marker gene \textit{aadA} (conferring resistance to the aminoglycoside antibiotics spectinomycin and streptomycin; Figure 2). As with this construct, \textit{nptII} mRNA accumulation could, at least in part, come from co-transcription with \textit{trnfM} (due to read-through caused by incomplete transcription termination; (36), we also generated a plastid transformation vector with the opposite orientation of the \textit{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 \textit{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 \textit{trnfM} and \textit{trnG} genes (Figure 2) and test for the presence of a homogeneous population of transgenic plastid genomes (homoplasmasy). 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 (\textit{nptII} and \textit{aadA}) into the \textit{trnfM}\textit{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
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 Prrn (21, 31). The Prrn 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 plasmid-type mitochondrial promoter was much lower than from the extremely strong Prrn 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 Prrn: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 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 (Styl/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. (B) Example of a seed assay to confirm homoplasmy of transplastomic lines produced with vectors pSR7 and pSR8. Total cellular DNA was digested with BamHI and hybridized to a radiolabeled probe (Styl/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.
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 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 (Prrn:nptII), in which nptII is driven by the strongest known plastid promoter, the ribosomal RNA operon promoter Prrn, 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.

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 Prrn: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 (Prrn: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 PatpA-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
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-32P-UTP labeled nascent RNAs were hybridized to filters containing dot-blotted sequences of the nptII gene and of the resident chloroplast genes rpoB, clpP, psbA and nptII. 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).
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 PatpA-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 PatpA-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 PatpA-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 rRNA 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 rRNA 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 cts-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 plastotransgenic experiments, the mitochondrial atpA promoter proved to be stronger than the resident chloroplast NEP promoters of cplP 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 these 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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