

# High diversity of plastidial promoters in *Arabidopsis thaliana*

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**Abstract** *Arabidopsis thaliana* is well established as a model plant in modern plant biology. However, remarkably few details are known about plastidial promoters in *Arabidopsis*. Here, we report on the identification and analyses of sequences at transcription start sites of selected genes. The genes encoded by the plastome of higher plants are transcribed by a plastid-encoded (PEP) and a nuclear-encoded RNA plastid polymerase (NEP). To discriminate between NEP and PEP promoters we compared the 5'-ends of transcripts from chlorophyll-deficient *Arabidopsis* plants, which were grown on prokaryotic translation inhibitor spectinomycin to inhibit biosynthesis of PEP, with those of untreated plants. Using 5'-RACE combined with enzymatic treatment of RNAs to recognize primary and secondary 5'-ends, we unambiguously identified transcription initiation sites of the *Arabidopsis accD*, *atpB*, *atpI*, *rpoB*, *rps4*, *rps15*, and *ycf1* genes. Comparison of plastidial promoters from tobacco and *Arabidopsis* revealed a high diversity, which may also apply to other plants. Furthermore, the diversity in individual promoter usage in different plants suggests that there are species-specific solutions for attaining control over gene expression in plastids.

**Keywords** *Arabidopsis* · Plastid transcription · Phage-type RNA polymerase · Nuclear-encoded RNA polymerase (NEP) · Plastid promoter

## Introduction

Chloroplasts, as the sites of photosynthesis, have a central role in the metabolism of plant cells (Gray 1993; López-Juez and Pyke 2005). Although many genes of the genome of the ancestral cyanobacteria have been transferred into the nucleus, plastomes still harbor genes for products that function primarily in photosynthesis and gene expression. Examples are the *rpo* genes that encode homologues of the bacterial RNA polymerase core subunits, which are complemented with nuclear encoded  $\sigma$ -factors to form the plastid-encoded plastid RNA polymerase (PEP; Shiina et al. 2005; Liere and Börner 2007). In surprising contrast to their bacterial ancestors, this RNA polymerase is not sufficient to transcribe all genes encoded by the rather small plastomes of higher plants. The existence of a second, plastid-localized, nuclear-encoded transcription activity (NEP, nuclear-encoded plastid RNA polymerase) was established by analyzing mutant and transplastomic plants, respectively, that transcribe plastid genes despite the lack of PEP (reviewed in Hess and Börner 1999; Weihe 2004; Liere and Börner 2007). These studies revealed three classes of plastidial genes. Generally, genes encoding components for the photosynthetic apparatus have PEP promoters (class I), whereas non-photosynthesis-related genes have promoters for both RNA polymerases (class II); few genes are transcribed exclusively from an NEP promoter (class III; Maliga 1998).

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Since PEP has evolved from a eubacterial-type RNA polymerase, it recognizes promoters which contain -35 (TTGaca) and -10 (TATAaT) consensus sequences typical of  $\sigma^{70}$ -type *E. coli* promoters (Reznikov et al. 1985; for reviews see Gruijsem and Tonkyn 1993; Link 1994; Hess and Börner 1999; Weihe and Börner 1999; Liere and Maliga 2001; Weihe 2004). NEP transcription activity is represented by an RNA polymerase similar to RNA polymerases of the T7 phage type (Lerbs-Mache 1993; Chang et al. 1999; Liere and Maliga 1999; Liere et al. 2004). Genes encoding organellar phage-type RNA polymerases (*RpoT*) have been found in several higher plant genomes (Liere and Börner 2007). One organellar phage-type RNA polymerase is targeted to plastids (RpoTp), a second is targeted into mitochondria (RpoTm), whereas a third type is found exclusively in dicots, and encodes an enzyme dually targeted into both mitochondria and plastids (RpoTmp; Weihe 2004; Khan 2005). Most NEP promoters consist of a core sequence (YRTA; type-Ia), similar to promoters of plant mitochondria (Liere and Maliga 1999, 2001; Weihe and Börner 1999; Binder and Brennicke 2003; Kühn et al. 2005). A subclass of NEP promoters shares a GAA-box motif upstream of the YRTA-motif (type-Ib; Kapoor and Sugiura 1999). Type-II NEP promoters, represented by dicot *clpP* promoters, lack these motifs and possess crucial sequences located downstream of the transcription initiation site (Weihe and Börner 1999; Liere and Maliga 2001). Furthermore, the existence of additional non-consensus NEP promoters (Pc) has been reported for the *rrn* operon in spinach, mustard, and Arabidopsis, and for the internal promoters of certain tRNAs (reviewed in Liere and Börner 2007).

Although Arabidopsis is well established as a model plant in modern plant biology, remarkably few details are known about its plastidial promoters. In addition to the PEP promoters *PpsbA-77* (Liere et al. 1995; Shen et al. 2001), *PrbcL-179*, *PpsbD-256*, *PpsbD-541*, *PtrnE-UUC*, *PtrnV-UAC* (Hanaoka et al. 2003), *PpsbD-946* (Hoffer and Christopher 1997; Hanaoka et al. 2003), *PpsaA-188* (Fey et al. 2005), *PpsaJ-37* (Nagashima et al. 2004), and *Prrn16-112* (Sriraman et al. 1998b), only two NEP promoters, *PclpP-58* (type-II; Sriraman et al. 1998a), and *Prrn16-139* (Pc; Sriraman et al. 1998b) have been identified (promoters are specified by the name of the gene and the position of the initiating nucleotide with respect to the start of the coding sequence or mature rRNA). Comparison of transcription in various plant species revealed striking differences in promoter usage for some plastome-encoded genes (e.g. *rrn16*; reviewed in Liere and Börner 2007). The use of different promoters and types of RNA

polymerases in expression of plastidial genes is not yet fully understood. Furthermore, identification of NEP promoters in Arabidopsis is an important prerequisite to analyze promoter specificities of the plastid-localized RpoTp and RpoTmp enzymes. Therefore, we investigated promoter usage using 5'-RACE combined with enzymatic treatment of RNAs to discriminate between primary (formed by transcription initiation) and secondary 5'-ends (formed by nucleolytic processing steps; Bensing et al. 1996; Kühn et al. 2005). Mutants or transplastomic plants lacking PEP activity have not been described in Arabidopsis. We therefore used leaves from chlorophyll-deficient Arabidopsis plants grown on spectinomycin as the material in our experiments (Zubko and Day 1998). Since the core subunits of PEP are synthesized on plastid ribosomes, treatment with spectinomycin, which inhibits plastidial translation (Moazed and Noller 1987), should lead to PEP-deficiency. Therefore, comparison of transcription initiation sites used in plastids of spectinomycin-treated and untreated material should allow for discrimination between NEP and PEP promoters. Here, we report on the analyses of sequences at transcription start sites of selected plastome-encoded genes. Although some plastidial promoter sequences are highly homologous across plant species, highly diverse promoter sequences and types indicate species-specific solutions in gaining control over plastidial gene expression.

## Materials and methods

### Plant material

*Arabidopsis thaliana* (ecotype Columbia) was grown on MS medium under a light–dark cycle of 8 h/16 h at 20°C. After 14 days, the seedlings were exposed to a light regime of 16 h light/8 h darkness. The leaves were harvested after 21 days for RNA isolation. Germinating Arabidopsis seeds on MS medium containing 500 mg ml<sup>-1</sup> spectinomycin dihydrochloride (Duchefa) generated chlorophyll-deficient plants, which were harvested after growing for 21 days on the same medium (Zubko and Day 1998). The light intensity was 150  $\mu\text{E s}^{-1} \text{m}^{-2}$ .

### RNA isolation and mapping of transcription initiation sites

The 5'-ends of organellar primary transcripts carry triphosphates, while processed transcripts have monophosphates at their 5'-ends. The latter can be selectively ligated to an RNA oligonucleotide (heswa-001; 5'-GAUAUGC GCGAAU UCCUGUAGAACGAA

CACUAGAAGAAA-3'), to which a forward primer (P1a; 5'-CGAATTCCTGTAGAACGAACACTAGAAG-3') anneals in a subsequent 5'-RACE step. 5'-ends of primary transcripts are ligated only after dephosphorylation through tobacco acid pyrophosphatase (TAP; Epicentre Biotechnologies). Thus, 5'-RACE yields products from TAP-treated RNA for both primary and processed transcripts, whereas without TAP treatment, products resulting from primary transcript termini are absent. Primary transcript 5'-ends are therefore identified by comparison of 5'-RACE products obtained from TAP-treated (+T) and non-treated RNA (-T).

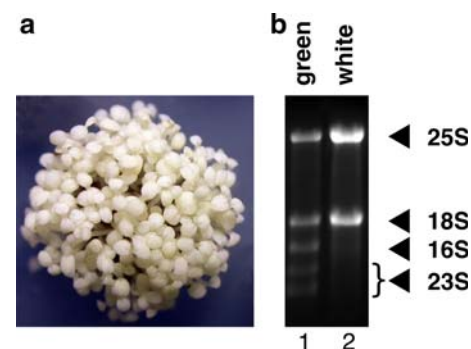
RNA was extracted from 21-day-old leaves and isolated plastids (Gruissem et al. 1986) using TRIzol (Invitrogen) following the manufacturer's protocol and subjected to TAP-treatment and 5'-RACE as detailed in Kühn et al. (2005). Starting from the coding regions, reverse gene-specific primers for cDNA synthesis and PCR were designed to span intergenic regions upstream of the tested genes in intervals of about 100–300 bp (Fig. S1 and S2). Relevant gene-specific primers are listed in Table 1. Adaptor primer P1a served as forward primer in PCR reactions. PCR reactions were analyzed on agarose gels. Products of interest were excised, purified, and ligated into pDrive (Qiagen). Bacterial clones containing the plasmid insert were identified by colony PCR with vector-specific primers, and PCR products were purified and sequenced. Obtained sequences were aligned and analyzed for heswa-linker/plastome sequence junctions which mark transcript 5'-ends.

## Results

### PEP-deficient Arabidopsis plants

With few exceptions, most NEP promoters are actively used, and therefore mapped, only in plastids of

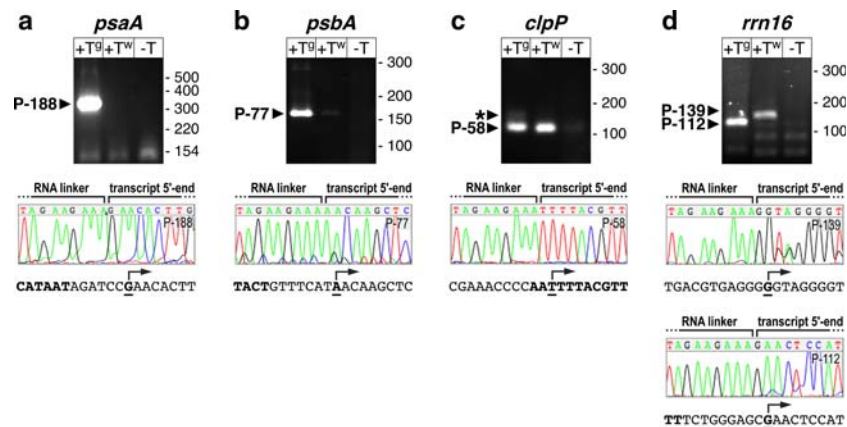
non-green tissues with no or reduced PEP activity (Shiina et al. 2005; Liere and Börner 2007). Hence, most NEP promoters in dicots were characterized in transplastomic tobacco plants lacking one of the PEP core components ( $\Delta rpo$ ; Hajdukiewicz et al. 1997; Serino and Maliga 1998). Thus far, plastid transformation to knock out *rpo* genes in Arabidopsis is not routinely feasible (Sikdar et al. 1998). To map Arabidopsis NEP promoters we blocked plastidial protein synthesis using the antibiotic spectinomycin, which generated chlorophyll-deficient plants lacking PEP (Zubko and Day 1998). On spectinomycin containing media Arabidopsis plants germinated and grew for about 10 days until their further development stopped (Fig. 1a). RNA isolated from these plants showed drastically reduced levels of plastidial ribosomal RNA (Fig. 1b), which indicated a severe loss in their translational competence. The deficiency in translation resulted in low or lacking PEP activity as evident from reduction or loss of transcripts generated by PEP (Fig. 2, *psaA*, *psbA*, *rrn16*; Fig. 4, *atpI*).



**Fig. 1** Chlorophyll-deficient Arabidopsis. Arabidopsis seeds germinated on MS medium containing spectinomycin generated chlorophyll-deficient plants (a). RNA isolated from 21 days old plants showed drastically reduced levels of plastidial ribosomal RNA (b, lane 1), when compared to the wild type (lane 2). Positions of ribosomal RNAs are indicated on the right

**Table 1** Gene specific primers used for cDNA synthesis and PCR reactions

Name	Sequence (5' – 3')	5' position
P1 <i>clpP</i>	GGTACTTTTGGAACGCCAATAGGC	71,857
P3 <i>atpB-a</i>	CGGTTATGCGTCCCATTATTCATC	54,537
P3 <i>atpB-b</i>	GTAAGCACTCGATTTCGTTGGTCC	54,253
P3 <i>atpI</i>	GTTTTGGATCCCACTAAACAAATCAC	14,861
P3 <i>psaA</i>	GGTTTAGCCCATTCCTCGAAAG	41,772
P3 <i>rps4</i>	GCCCTCGGTAACGTGACATAAAG	45,810
P3 <i>ycf1</i>	GGGCCCTATGGAAAATGTGG	109,524/129,125
P4 <i>rpoB-a</i>	TTTTTGAATTTCCCATTTATCCGTTTA	26,332
P4 <i>rps15</i>	TCCTTCCCCTTTTAATATGAATTGAAAGATAT	123,572
P4 <i>rrn16</i>	TAGTTGCATTACTTATAGCTTCCTTCCTCG	100,978/137,642
P5 <i>saccD</i>	AGAGTAAGCAAAACATATCGATGCAA	56,965
P5 <i>psbA</i>	TCCAGTTACAGAAGCGACCCCATAG	1,384



**Fig. 2** Identification of *psaA*, *psbA*, *clpP*, and *rrn16* promoters in Arabidopsis plastids. Arabidopsis RNA isolated from *green* (+T<sup>g</sup>) and *white* leaf tissue (+T<sup>w</sup>) 5'-ligated to an RNA-linker after TAP-treatment and, as a control, untreated RNA (-T) were subjected to RT-PCR with *psaA* (a), *psbA* (b), *clpP* (c), and *rrn16* (d) gene-specific primers (Table 1). Products were separated on agarose gels alongside molecular weight markers; sizes are indicated in base pairs. Please note that the PCR fragments contain

#### Identification of transcript initiation sites of plastidial genes

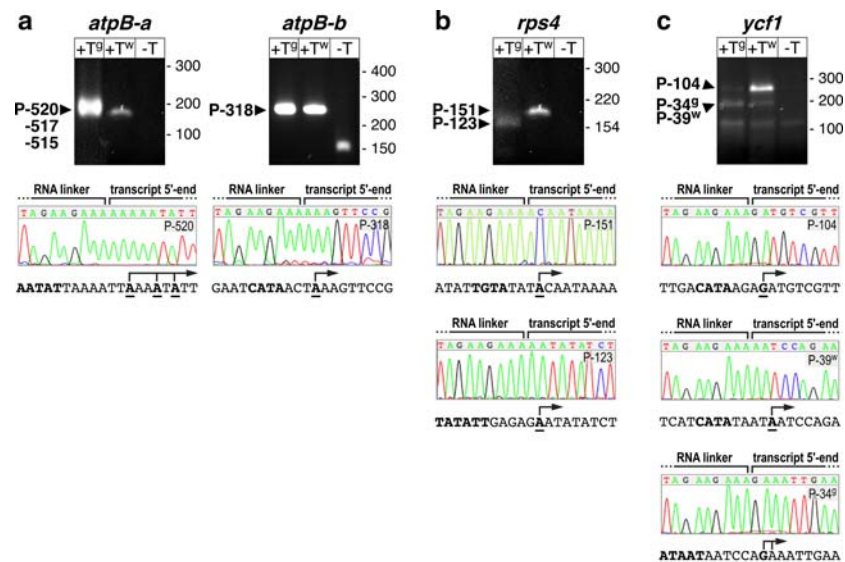
To precisely determine transcript initiation sites of the *psaA*, *psbA*, *atpB*, *atpI*, *clpP*, *rrn16*, *rps4*, *rps15*, *accD*, *rpoB*, and *ycfI* genes we used a previously described 5'-RACE technique selectively detecting 5'-ends of primary transcripts (Bensing et al. 1996; Miyagi et al. 1998; Kühn et al. 2005). To distinguish between PEP and NEP generated transcripts, we compared initiation sites of RNA isolated from green (+T<sup>g</sup>) and chlorophyll-deficient spectinomycin-treated tissue (+T<sup>w</sup>). To prove the concept, we mapped the transcription initiation sites of known PEP and NEP promoters. Analysis of *psaA* transcripts revealed a distinct PCR fragment of 305 bp, which was only amplified from TAP-treated RNA isolated from green tissue (+T<sup>g</sup>) but neither from TAP-treated RNA isolated from white (+T<sup>w</sup>) nor from untreated RNA (-T, Fig. 2a). This was therefore concluded to correspond to a primary PEP transcript. As described by Fey et al. (2005), sequence analysis placed the native *psaA* 5'-end at position -188 with respect to the translation initiation site (+1). Similarly, we located the exact 5'-end of native *psbA* transcripts at position -77 (Fig. 2b), consistent with earlier reports (Liere et al. 1995; Shen et al. 2001). Interestingly, a weak PCR signal was also detected in +T<sup>w</sup> samples. Sequence analysis revealed the same initiation site *PpsbA*-77 as detected in +T<sup>g</sup> samples (data not shown). This suggests that some residual PEP transcripts are still present in spectinomycin-treated plants, only detectable by the sensitive PCR method. As expected from data on

additional 31 bp of the *heswa*-linker that was ligated to the RNA. *Arrows* mark bands corresponding to primary transcript 5'-ends and labeled with the position in respect to the translation initiation site or mature rRNA (+1). Chromatograms below display sequences at the ligation sites of cloned 5'-RACE products. Additionally, sequences of the promoter regions with putative promoter motifs marked in *bold face letters* are given. Transcription initiation sites are *underlined* and marked by *bent arrows*

*clpP* expression published by Sriraman et al. (1998a), we were able to detect transcript 5'-ends from the strong type-II *PclpP*-58 NEP promoter in both +T<sup>g</sup> and +T<sup>w</sup> assays (Fig. 2c). However, although a PCR fragment of the size expected for a transcript initiated from the PEP promoter *PclpP*-115 has been detected (Fig. 2c, +T<sup>g</sup>, asterisk), we did not succeed in determining the sequence. As described for *rrn16* (Sriraman et al. 1998b), transcripts initiated at the PEP promoter *Prrn16*-112 were mapped in +T<sup>g</sup>, as well as transcripts initiated at the Pc promoter *Prrn16*-139 in +T<sup>w</sup> samples (Fig. 2d). Therefore, we concluded that we were able to distinguish between NEP and PEP promoters in our assays.

The tobacco *atpB* gene is transcribed from at least four promoters (Hajdukiewicz et al. 1997). In Arabidopsis, however, only two initiation sites were found (Fig. 3a). One clusters at positions -520/-517/-515 strongly detectable in green RNA samples (panel *atpB*-a; +T<sup>g</sup>) and is preceded by eukaryotic -35/-10 promoter consensus motifs of PEP promoters (Fig. 5a). As observed in the *psbA* assays, frail residual transcripts originating from this initiation site were also detectable in RNAs of white leaf tissue (panel *atpB*-a; +T<sup>w</sup>). Using a different primer (*P3atpB*-b), we detected a PCR fragment in both RNAs placing a native RNA 5'-end at position -318 (Fig. 3a, panel *atpB*-b). This initiation site is preceded by a CATA sequence showing similarity to the YRTA-consensus motif of type-I NEP promoters (Fig. 5b). These data indicate that *atpB* is transcribed by both NEP and PEP in green tissue.





**Fig. 3** Arabidopsis *atpB*, *rps4*, and *ycf1* genes are transcribed from both NEP and PEP promoters. Arabidopsis RNA isolated from *green* (+T<sup>g</sup>) and *white* leaf tissue (+T<sup>w</sup>) 5'-ligated to an RNA-linker after TAP-treatment and, as a control, untreated RNA (-T) were subjected to RT-PCR with *atpB* (**a**), *rps4* (**b**), and *ycf1* (**c**) gene-specific primers (Table 1). In case of *atpB* two primers were used, P3*atpB*-a in *atpB*-a and P3*atpB*-b in *atpB*-b. PCR-fragments representing the different transcript 5'-ends of PCR-fragments of *ycf1* in *green* and *white* tissue are marked with

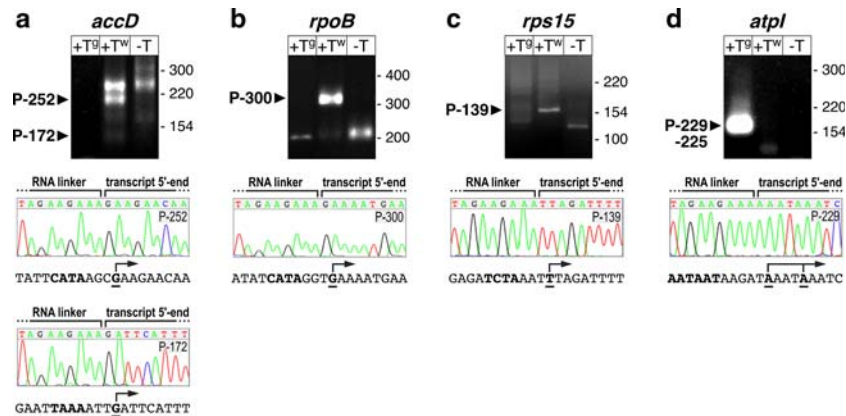
Transcription initiation sites with upstream-located PEP (Fig. 5a) and NEP promoter elements (Fig. 5b) were also found upstream of *rps4* (Fig. 3b). However, while the PEP initiation site *Prps4*-123 was only detectable in green tissue (+T<sup>g</sup>), transcripts initiated from *Prps4*-151 were only evident in RNA of white leaf tissue (+T<sup>w</sup>), indicating that *rps4* is transcribed by PEP but not by NEP in green tissue.

Characterization of *ycf1* 5'-ends revealed three transcription start sites at positions -34, -39, and -104 (Fig. 3c). The *Pycf1*-104 initiation site was detected in both RNA samples (+T<sup>g</sup> and +T<sup>w</sup>; 255 bp). Different 5'-ends, however, were identified by sequence analyses of the smaller PCR fragments (190 bp in +T<sup>w</sup>; 185 bp in +T<sup>g</sup>). The 190-bp fragment, which was amplified in white leaves, revealed an initiation site at position -39 (*Pycf1*-39; in Fig. 3c marked with P-39<sup>w</sup>). However, the 185-bp fragment detected in green leaves placed a 5'-end at position -34 (*Pycf1*-34; in Fig. 3c indicated as P-34<sup>g</sup>). Sequences upstream of *Pycf1*-39 and *Pycf1*-104 show typical CATA motifs of type-I NEP promoters. The *Pycf1*-39 promoter sequence is highly conserved to the sole *ycf1* promoter in tobacco (Nt*Pycf1*-41; Hajdukiewicz et al. 1997) and was classified into the group of type Ib promoters (Fig. 5b). *Pycf1*-34 active in green leaves, however, displays typical -35/-10 motifs of PEP

the letters 'g' and 'w'. Products were separated on agarose gels alongside molecular weight markers; sizes are indicated in base pairs. Arrows mark bands corresponding to primary transcript 5'-ends and labeled with the position in respect to the translation initiation site or mature rRNA (+1). Chromatograms below display sequences at the ligation sites of cloned 5'-RACE products. Additionally, sequences of the promoter regions with putative promoter motifs marked in *bold face letters* are given. Transcription initiation sites are *underlined* and marked by *bent arrows*

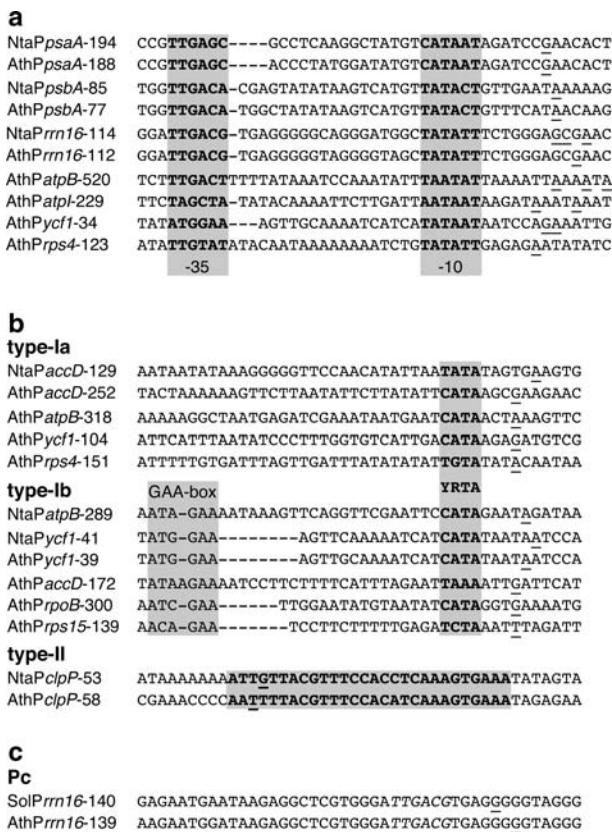
promoters upstream of its initiation site, suggesting a polymerase switch from NEP to PEP comparing white and green tissue in this distinct *ycf1* upstream region.

Analysis of RNAs synthesized from the *accD* gene (Fig. 4a) revealed two PCR fragments of 170 and 240 bp which were amplified from TAP-treated RNA from white leaves (+T<sup>w</sup>) and untreated cellular RNA (-T) indicating processed RNA 5'-ends. Sequence analysis of two additional bands of 94 and 174 bp in size detected in +T<sup>w</sup> but neither in +T<sup>g</sup>, nor in -T samples placed native *accD* 5'-ends at positions -172 and -252. CATA (*PaccD*-252) and TAAA (*PaccD*-172) sequence motifs are located immediately upstream, showing similarity to the YRTA-consensus motif of type-I NEP promoters. In case of *PaccD*-172, an ATA-AGAA-motif found immediately upstream allowed sub-classification of this promoter into the group of type-Ib NEP promoters, whereas *PaccD*-252 belongs to the group of type-Ia NEP promoters (Fig. 5b). Interestingly, the processing site yielding the 240-bp PCR fragment indicates transcriptional initiation further upstream. However, TAP assays spanning the *rbcL*-*accD* intergenic region did not reveal further 5'-ends of primary transcripts suggesting additional initiation of transcription within or upstream of the *rbcL* gene (data not shown).



**Fig. 4** Arabidopsis *accD*, *rpoB*, *rps15*, and *atpI* genes are transcribed from NEP or PEP promoters. Arabidopsis RNA isolated from green (+T<sup>g</sup>) and white leaf tissue (+T<sup>w</sup>) 5'-ligated to an RNA-linker after TAP-treatment and, as a control, untreated RNA (-T) were subjected to RT-PCR with *accD* (**a**), *rpoB* (**b**), *rps15* (**c**), and *atpI* (**d**) gene-specific primers (Table 1). Products were separated on agarose gels alongside molecular weight markers; sizes are indicated in base pairs. Arrows mark bands

corresponding to primary transcript 5'-ends and labeled with the position in respect to the translation initiation site or mature rRNA (+1). Chromatograms below display sequences at the ligation sites of cloned 5'-RACE products. Additionally, sequences of the promoter regions with putative promoter motifs marked in *bold face letters* are given. Transcription initiation sites are *underlined* and marked by *bent arrows*



**Fig. 5** Synopsis of PEP (**a**) and type-Ia, type-Ib, type-II NEP (**b**), and Pc promoters (**c**). Conserved -35/-10 boxes, YRTA motifs and GAA-box are *marked*; mapped transcription initiation sites are *underlined*. Tobacco promoter sequences are displayed according to Hajdukiewicz et al. (1997), Sriraman et al. (1998a), Vera and Sugiura (1995), and Meng et al. (1988); spinach promoter sequences according to Iratni et al. (1997)

In case of the *rpoB* operon encoding three subunits of the plastid-encoded plastid RNA polymerase (PEP), PCR analysis revealed in +T<sup>w</sup> assays a prominent fragment of 328 bp (Fig. 4b). This places a transcription start site at position -300. In contrast to the tobacco type-Ia NtPrpoB-345 promoter (Liere and Maliga 1999), CATA and ATCGAA sequence motifs immediately upstream of the initiation site suggest a classification of PrpoB-300 as type Ib NEP promoter (Fig. 5b).

Correspondingly, characterization of *rps15* transcript 5'-ends revealed a sole initiation site of 139 bases upstream of the translation initiation site, observable only in RNAs of white leaf tissue (Fig. 4c; +T<sup>w</sup>). Directly upstream of the transcription initiation site, a core TCTA and a further upstream-located ACAGAA sequence motif suggest a categorization of Prps15-139 into the group of type-Ib NEP promoters (Fig. 5b).

In case of *atpI*, we found transcription initiation sites at positions -229 and -225, detectable only in assays with RNA from green leaves (Fig. 4d; +T<sup>g</sup>). The directly upstream located -35/-10 promoter elements assigned PatpI-229 to the group of PEP promoters (Fig. 5a).

**Discussion**

Remarkably few details are known about plastidial promoters in *Arabidopsis thaliana*. Here, we report on the identification and analyses of sequences at transcription initiation sites of selected plastidial genes. To

discriminate between NEP and PEP promoters we compared RNAs from chlorophyll-deficient Arabidopsis plants grown on spectinomycin (Fig. 1) and untreated material (Zubko and Day 1998). Transcription initiation sites were mapped using 5'-RACE combined with enzymatic treatment of RNAs to discriminate between primary and secondary 5'-ends (Bensing et al. 1996; Kühn et al. 2005). By mapping known NEP and PEP promoters (Fig. 2) we were able to prove that we could distinguish between the two promoter types. Interestingly, residual amounts of some PEP-derived transcripts (e.g. *psbA*, Fig. 2b) were detectable in RNAs from spectinomycin-treated plants. This was also reported for spectinomycin-treated albino *Brassica napus* plants, which, however, showed no detectable amounts of D1 protein (Zubko and Day 1998). Similarly, RNA gel analysis revealed strongly reduced but still detectable amounts of 16S rRNA, which are likely to be generated by the Pc-promoter *Prrn16-139* active in white tissues (Fig. 2d).

In this study, we added transcription initiation sites for *atpB*, *atpI*, *rps4*, *rps15*, *rpoB*, *accD*, and *ycf1* to the portfolio of plastidial promoters in Arabidopsis (Figs. 3, 4). We scanned the upstream regions of genes for 5'-ends of primary transcripts using a primer walking approach with several reverse primers in intervals of 100–300 bp (Fig. S1 and S2). Our analyses determined PEP promoters (Fig. 5; *PatpB-520*, *PatpI-229*, *Prps4-123*, *Pycf1-34*) as well as NEP promoters (Fig. 5; *PaccD-252*, *PaccD-172*, *PatpB-318*, *PrpoB-300*, *Prps4-151*, *Prps15-139*, *Pycf1-39*, *Pycf1-104*). Although our scan covered a region of about up to position –800, previous data on putative Arabidopsis *rpoB* (–563; Inada et al. 1997) and *accD* (–89; Hanaoka et al. 2005) transcription initiation sites derived by primer extension analyses could not be confirmed (see Fig. S1 for data on *rpoB*; data for *accD* not shown). However, this does not rule out the possibility of even further upstream located promoters, as was shown for the tobacco *ycf2* gene (NtaPycf2-1577; Hajdukiewicz et al. 1997).

The tobacco *atpB* gene is transcribed from at least three NEP (NtaPatpB-255, –502/–488, –611) and two PEP promoters (NtaPatpB-289, –329; Hajdukiewicz et al. 1997). However, similar to maize (NEP ZmaP-*atpB*-601 and PEP ZmaPatpB-298; Silhavy and Maliga 1998) only one PEP (*PatpB-520*) and one NEP promoter (*PatpB-318*) are driving this gene in Arabidopsis. Although sequences of tobacco NtaPatpB-488 and NtaatpB-611 PEP promoters are conserved within the upstream region of the Arabidopsis *atpB* gene, they are not utilized. This is reminiscent to the diverse promoter usage observed for *clpP* and *rrn16*. Although the

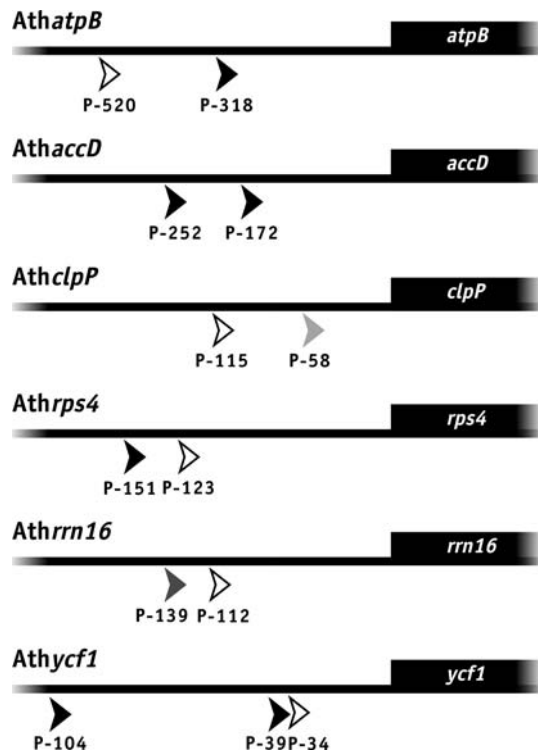
promoter regions of both genes are well conserved, different types of RNA polymerases and *cis*-elements are used in distinct plants (Baeza et al. 1991; Sriraman et al. 1998a; reviewed in Liere and Börner 2007).

Interestingly, *ycf1* is transcribed from a highly conserved NEP promoter in tobacco and Arabidopsis (Fig. 5b; *Pycf1-39*, *Ntaycf1-41*). However, apart from an additional NEP promoter (*Pycf1-104*) active in both green and white leaves, a PEP promoter (*Pycf1-34*) located at the NEP promoter position of *Pycf1-39* takes over transcription in green leaves. Besides the *rrn16* Pc and PEP promoters in Arabidopsis, this is the second report of a defined DNA sequence serving as a promoter for both, NEP and PEP.

However, upstream sequences of some genes, such as *accD*, *atpI*, *rps4*, and *rpoB*, are not highly conserved. Nevertheless, the *accD* and *rpoB* genes of both tobacco and Arabidopsis are transcribed by NEP. Although one NEP promoter precedes the tobacco *accD* gene (NtaPaccD-129; Hajdukiewicz et al. 1997), two NEP promoters were found to transcribe the Arabidopsis *accD* gene (*PaccD-172*, –252). However, *rpoB* seems to be transcribed from a sole NEP promoter in both plants (*PrpoB-300*, NtaPrpoB-345; Liere and Maliga 1999). Similarly, as formerly predicted from studies in barley (Hess et al. 1993) and Arabidopsis mutants (Nagashima et al. 2004), the Arabidopsis *rps15* is transcribed by NEP from *Prps15-139*. The spinach *rps4* gene was shown to be transcribed by PEP (Tahar et al. 1986) which seems also to be the case in Arabidopsis (*Prps4-123*). However, an additional NEP promoter (*Prps4-151*) was also detected in white, spectinomycin-treated Arabidopsis leaves. Conversely, *atpI* in tobacco is transcribed by both NEP and PEP (NEP NtaPatpI-207, PEP NtaPatpI-130; Miyagi et al. 1998), whereas transcription of the Arabidopsis *atpI* gene is driven by a sole PEP promoter (*PatpI-229*). The diversity of individual promoter usage in different plants therefore suggests species-specific solutions in controlling gene expression in plastids.

Although genes exist that are transcribed from a single promoter, transcription of plastidial genes and operons by multiple promoters seems to be a rather common feature (Fig. 6). Furthermore, a number of genes are reported to be co-transcribed with other genes within an operon and to additionally possess an individual promoter upstream of their coding region (e.g. *trnG* and *psbA*; reviewed in Liere and Börner 2007). The role of most multiple promoters upstream of plastidial genes and operons is not fully understood; however, some are well characterized. The blue-light-responsive promoter (BRLP) of *psbD-psbC* is thought to differentially maintain the ability to re-synthesize





**Fig. 6** Arabidopsis genes with multiple promoters. Schematic synopsis which shows the multiple PEP and NEP promoters of Arabidopsis genes. Boxes represent genes, while open arrowheads denote PEP promoters, filled black arrowheads type-I NEP promoters, filled light gray arrowhead the *PclpP*-58 type-II NEP promoter, and filled dark gray arrowhead the *Prrn16*-139 Pc promoter. The promoters are named based on their position in respect to the translation initiation site (+1)

and replace damaged D2 and CP43 photosystem components in mature chloroplasts (Christopher and Mullet 1994). It has been shown that two promoters are responsible for differential transcription of the *petE* operon in maize. While monocistronic *petE* transcripts accumulate under light conditions, usage of a further upstream located promoter results in polycistronic transcripts covering the *petL-petE-psaJ* gene cluster in the dark (Haley and Bogorad 1990).

In spite of the observed diversity between plastidial genes of Arabidopsis and other species of higher plants, our data support also the existence of common themes in promoter usage that have been deduced mainly from studies on transcription in tobacco plastids. Mixed NEP and PEP promoters typically are found upstream of housekeeping genes which need to be transcribed during full plastidial development (Maliga 1998). Consequently, both promoter types are believed to differentially express their cognate gene during plant development (reviewed in Liere and Maliga 2001). NEP promoters seem to be generally recognized in youngest and non-green tissues early in

plant development, while PEP takes over in maturing photosynthetically active chloroplasts (Bisanz-Seyer et al. 1989; Baumgartner et al. 1993; Hajdukiewicz et al. 1997; Kapoor et al. 1997; Emanuel et al. 2004). Large spurious transcripts initiated by NEP cover the entire plastome in tobacco  $\Delta rpo$  mutants lacking PEP, suggesting that besides selective promoter utilization, post-transcriptional processes also determine the transcript pattern of plastids (Krause et al. 2000; Legen et al. 2002). Data derived from analyses of the developmental gradient in maize leaves suggest that, as plastids mature, the stability of transcripts generated by NEP declines, although the transcriptional activity by NEP increases (Cahoon et al. 2004). However, exclusively NEP-transcribed regions encoding housekeeping functions, such as the *rpoB* operon and *rps15* gene, suggest that NEP is important for proper gene expression and regulation also in mature chloroplasts. Indeed, NEP and PEP are active throughout leaf development in Arabidopsis, although PEP seems to play a major role in mature leaves (Demarsy et al. 2006; Zoschke et al. 2007). Interestingly, exclusively PEP-transcribed genes code for proteins with a role in photosynthesis. As the major active polymerase in mature chloroplasts, present data point to PEP as a prominent target for regulation signals including redox control, not yet determined for NEP (for review see Forsberg et al. 2001; Liere and Maliga 2001; Pfannschmidt and Liere 2005). Since plants that turn to a parasitic lifestyle lose photosynthetic genes as well as PEP promoters (Wolfe et al. 1992a, b; Krause et al. 2003; Berg et al. 2004), transcription and regulation of gene expression by PEP might be connected to photosynthesis. The knowledge of plastidial promoters in Arabidopsis will help to define the role of both the NEP and PEP RNA polymerases in plant development in future experiments.

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