**Arabidopsis** Phage-Type RNA Polymerases: Accurate in Vitro Transcription of Organellar Genes

Kristina Kühn,1,2 Alexandra-Viola Bohne,1 Karsten Liere, Andreas Weihe, and Thomas Börner

Institute of Biology (Genetics), Humboldt University, D-10115, Berlin, Germany

The T7 bacteriophage RNA polymerase (RNAP) performs all steps of transcription, including promoter recognition, initiation, and elongation as a single-polypeptide enzyme. *Arabidopsis thaliana* possesses three nuclear-encoded T7 phage-type RNAPs that localize to mitochondria (RpoTm), plastids (RpoTp), or presumably both organelles (RpoTmp). Their specific functions are as yet unresolved. We have established an in vitro transcription system to examine the abilities of the three *Arabidopsis* phage-type RNAPs to synthesize RNA and to recognize organellar promoters. All three RpoT genes were shown to encode transcriptionally active RNAPs. RpoTmp displayed no significant promoter specificity, whereas RpoTm and RpoTp were able to accurately initiate transcription from overlapping subsets of mitochondrial and plastidial promoters without the aid of protein cofactors. Our study strongly suggests RpoTm to be the enzyme that transcribes most, if not all, mitochondrial genes in *Arabidopsis*. Intrinsic promoter specificity, a feature that RpoTm and RpoTp share with the T7 RNAP, appears to have been conserved over the long period of evolution of nuclear-encoded mitochondrial and plastidial RNAPs. Selective promoter recognition by the *Arabidopsis* phage-type RNAPs in vitro implies that auxiliary factors are required for efficient initiation of transcription in vivo.

**INTRODUCTION**

Unlike all RNA polymerases (RNAPs) of prokaryotic and eukaryotic organisms, the T3, T7, and SP6 bacteriophage RNAPs are single-polypeptide-chain enzymes. The comprehensively studied T7 RNAP can recognize specific promoter sequences, correctly initiate transcription, and catalyze transcript elongation until termination unaided by auxiliary proteins (reviewed in Cheetham, 2000). In mitochondria of the budding yeast *Saccharomyces cerevisiae* (Greenleaf et al., 1986; Masters et al., 1987), mammals (Tiranti et al., 1997; Falkenberg et al., 2002; Gaspari et al., 2004), and other eukaryotic organisms (Cermakian et al., 2004), a nuclear-encoded T7 phage-type RNAP has replaced the ancestral bacterial-type RNAP. Transcription initiation in the mitochondria of yeast and mammals, however, depends on the transcriptional cofactor mtTFB (MTF1, TFBM), which is related to rRNA dimethyladenosine transferases (Winkley et al., 1985; Schinkel et al., 1987; Falkenberg et al., 2002; McCulloch et al., 2002; Matsunaga and Jaehning, 2004b). Mammalian mitochondrial RNAP additionally requires the high-mobility group-box protein mtTFB (TFAM) for promoter recognition (Fisher and Clayton, 1985, 1988; Falkenberg et al., 2002; Gaspari et al., 2004). Recent in vitro studies unexpectedly revealed promoter specificity of the yeast mitochondrial transcription machinery to be conferred by the core RNAP rather than mtTFB (Matsunaga and Jaehning, 2004b).

Unlike mitochondria, plastids have retained genes for the core subunits of a bacterial-type RNAP called PEP (for plastid-encoded plastid RNAP). In addition to PEP, one or more nuclear-encoded plastid RNAP (NEP) is required for transcription in plastids of higher plants (Liere and Maliga, 1999; Bligny et al., 2000). NEP activity is probably represented by phage-type RNAPs (reviewed in Hess and Börner, 1999).

In contrast with fungi and animals, higher plants possess more than one T7 phage-type RNAP gene (*RpoT*; RNA polymerase of the T7/T3 type). *RpoT* genes have been identified in the nuclear genomes of various angiosperms (Hess and Börner, 1999; Weihe, 2004; Shilina et al., 2005; Liere and Börner, 2007) and in the moss *Physcomitrella patens* (Kabeya et al., 2002; Richter et al., 2002). According to in vitro and in vivo import studies, the small family of three *RpoT* genes in *Arabidopsis thaliana* encodes a mitochondrial RNAP (RpoTm), a plastidial enzyme (RpoTp), and a small polypeptide (RpoTmp) suggested to be imported into both mitochondria and plastids (RpoTmp) (Hedtke et al., 1997, 1999, 2000). The conservation of functionally critical amino acid positions of the T7 enzyme (McAllister and Raskin, 1993; Sousa et al., 1993) in RpoTm, RpoTp, and RpoTmp as well as in other plant RpoT polypeptides (Hess and Börner, 1999) and nonspecific transcription activity of recombinant RpoTmp in vitro (Hedtke et al., 2000) argue for *RpoT* gene products to function in organellar RNA synthesis. However, distinct roles of RpoTm and RpoTp in mitochondria and of RpoTp and RpoTmp in plastids are yet to be assigned.

Plant mitochondria transcribe their genomes from numerous promoters (Tracy and Stern, 1995), many of which contain a YRTA, RRTA, or ATTA motif (Y = T or C and R = A or G) immediately upstream of the initiating nucleotide (see Fey and Marechal-Drouard [1999] for a summary of earlier studies; Kühn...
et al., 2005). The majority of plastid NEP promoters also contain a core YRTA motif (class Ia NEP promoters; Hess and Börner, 1999), thus resembling mitochondrial promoters. A subclass of NEP promoters share a GAA-box motif upstream of the YRTA-motif, which was shown to be important in transcription from the tobacco (Nicotiana tabacum) PatpB-289 NEP promoter (class Ib; Kapoor and Sugiiura, 1999). Class II NEP promoters, however, lack these motifs, with crucial sequences located downstream of the transcription initiation site (Sriraman et al., 1998). A recent study of transgenic tobacco overexpressing an RpoTp cDNA revealed enhanced transcription from a subset of class I NEP promoters, suggesting RpoTp to be involved in transcription from these promoters (Liere et al., 2004).

The unique situation in Arabidopsis with the availability of various types of mitochondrial and plastidial promoters (Kühn et al., 2005; Swiatecka-Hagenbruch et al., 2007) and of three phage-type RNAPs (Hedtke et al., 2000) localizing to mitochondria and/or plastids prompted us to reconstitute a defined homologous in vitro transcription system to characterize and compare the activities of plant phage-type RNAPs. RpoTmp displayed no significant promoter specificity while showing high nonspecific transcription activity. By contrast, RpoTm and RpoTp were able to accurately initiate transcription from overlapping subsets of mitochondrial and plastidial promoters on supercoiled DNA templates without auxiliary factors, therefore retaining a characteristic feature of the T7 RNAP.

RESULTS

Three Active Phage-Type RNAPs in Arabidopsis

The Arabidopsis phage-type RNAPs RpoTm, RpoTmp, and RpoTp fused to an N-terminal thioredoxin-hexahistidine tag were expressed in Escherichia coli. Soluble recombinant RNAP enzymes were enriched from bacterial extracts (Figure 1A) and their RNAP activities compared in in vitro transcription assays with nonspecific DNA templates. All three enzymes were capable of catalyzing RNA synthesis (Figures 1B and 1C), indicating that all three RpoT genes encode functional RNAPs. RpoTmp overall exhibited considerably higher RNA synthesis rates than RpoTm and RpoTp and showed an 18-fold stimulation of its activity in the presence of a supercoiled compared with a linear plasmid template, as opposed to only a twofold stimulation of RpoTm and RpoTp (Figure 1B). Further assays tested the response of RpoT enzyme activities to RNAP inhibitors. Resembling T7 phage RNAP activity, RpoT-driven RNA synthesis was sensitive to actinomycin, a general inhibitor of transcription, but was essentially insensitive to rifampicin concentrations that efficiently inhibited E. coli RNAP (Figure 1C). A similar response to these drugs has been described for an undefined phage-type RNAP activity prepared from tobacco plastids that recognizes class I NEP promoters (Liere and Maliga, 1999). The observed differences in resistance patterns between the recombinant RpoT and E. coli enzymes and the below documented nonidentical promoter specificities of RpoTm, RpoTmp, and RpoTp (Figure 4) substantiated the in vitro transcription activities exhibited by RpoT preparations to be due to the recombinant enzymes rather than residual RNAP from the bacterial expression host.

Figure 1. Three Active Phage-Type RNAPs in Arabidopsis.

(A) RpoTm, RpoTmp, and RpoTp lacking the TargetP-predicted transit peptides (Emanuelsson et al., 2000) of 42, 104, and 95 amino acids, respectively, and fused N-terminally to a thioredoxin-hexahistidine tag were expressed in E. coli. Soluble recombinant RNAP enzymes were enriched from bacterial extracts (Figure 1A) and their RNAP activities compared in in vitro transcription assays with nonspecific DNA templates. All three enzymes were capable of catalyzing RNA synthesis (Figures 1B and 1C), indicating that all three RpoT genes encode functional RNAPs. RpoTmp overall exhibited considerably higher RNA synthesis rates than RpoTm and RpoTp and showed an 18-fold stimulation of its activity in the presence of a supercoiled compared with a linear plasmid template, as opposed to only a twofold stimulation of RpoTm and RpoTp (Figure 1B). Further assays tested the response of RpoT enzyme activities to RNAP inhibitors. Resembling T7 phage RNAP activity, RpoT-driven RNA synthesis was sensitive to actinomycin, a general inhibitor of transcription, but was essentially insensitive to rifampicin concentrations that efficiently inhibited E. coli RNAP (Figure 1C). A similar response to these drugs has been described for an undefined phage-type RNAP activity prepared from tobacco plastids that recognizes class I NEP promoters (Liere and Maliga, 1999). The observed differences in resistance patterns between the recombinant RpoT and E. coli enzymes and the below documented nonidentical promoter specificities of RpoTm, RpoTmp, and RpoTp (Figure 4) substantiated the in vitro transcription activities exhibited by RpoT preparations to be due to the recombinant enzymes rather than residual RNAP from the bacterial expression host.
RpoTm Recognizes Mitochondrial Promoters in Vitro

To elucidate distinct transcriptional roles of RpoTm, RpoTmp, and RpoTp in plant organelles, in vitro experiments investigating the transcription of organellar genes by these enzymes were required. To study transcription from mitochondrial promoters in vitro, DNA templates were constructed by inserting promoter regions of the *Arabidopsis* mitochondrial DNA into pKL23 (Liere and Maliga, 1999) upstream of the two bacterial ρ-independent terminator sequences hisa (Barnes and Tuley, 1983) and thra (Gardner, 1982) (Figure 2B). Promoters were selected based on in vivo transcription initiation sites determined previously for several mitochondrial genes in *Arabidopsis* (Kühn et al., 2005). In vitro RNA synthesis driven by a plant phage-type RNAP has been described earlier to efficiently stop at hisa and thra (Liere and Maliga, 1999). When providing a circular pKL23 derivative as template, transcription initiated at the introduced promoters should be terminated specifically at hisa and/or thra, thereby generating RNA products of distinct lengths. In vitro transcription was reconstituted from individual recombinant RpoT enzymes and circular (supercoiled) or linear DNA templates.

The promoters used in our transcription assays are listed in Table 1. Promoters and their corresponding transcription initiation sites are specified with the gene name and the position of the initiating nucleotide with respect to the start of the coding sequence or mature rRNA or tRNA.

Figure 2A shows the PAGE analysis of RNAs synthesized in vitro by RpoTm from supercoiled pKL23- atp6-1-A containing the promoters Patp6-1-156 and Patp6-1-200, which each display frequent mitochondrial promoter sequence motifs. Transcription from pKL23- atp6-1-A produced two major discrete RNA products of apparent lengths of ~300 and 370 nucleotides (Figure 2A; black arrows), as expected if transcription initiated at Patp6-1-200. While the upper indicated band (Figure 2A) resulted from transcription termination at thra, termination at hisa produced an RNA migrating as a double band. Since for several plasmid templates used in subsequent experiments, transcripts ending at hisa appeared as multiple bands, the latter presumably resulted from secondary structure formation at the transcript 3’ end or termination at multiple adjacent nucleotides at hisa. The high molecular weight signals that are visible at the top of the autoradiograph may in part be attributed to transcripts initiated nonspecifically on the plasmid template at sequences other than the atp6-1 promoters; another part likely represent RNAs not terminated at hisa and thra. Nonspecific high molecular weight transcripts were likewise seen in in vitro transcription studies with mitochondrial extracts (Hanic-Joyce and Gray, 1991; Rapp and Stern, 1992; Binder et al., 1995). The minor signals indicated by gray arrows and marked with asterisks were assumed to correspond to differently migrating major transcripts rather than additional defined RNA 5’ ends, as no discrete fragments other than those resulting from initiation at Patp6-1-200 were detected by 5’ rapid amplification of cDNA ends (RACE) (Figure 3).

Additional experiments using supercoiled templates pKL23-trnM and pKL23-trnM26 investigated the initiation at promoters PtmnM-98 and PtmnM26-893 (Figure 2). While the sequence around the transcription initiation site of PtmnM-893 is nearly identical to that of Patp6-1-156, PtmnM-98 displays elements of both transcription initiation at Patp6-1-156, Patp6-1-200, PtmnM-98, or PtmnM26-893 followed by termination at hisa or thra (arrowheads) are indicated by horizontal black arrows labeled with the respective RNA lengths. Annealing sites of primers P2hisa (2) and P3hisa (3) employed for transcript 5’ end mapping are marked. nt, nucleotides.

Figure 2. In Vitro Transcription from Mitochondrial Promoters by RpoTm.

(A) RpoTm was assayed for promoter-specific transcription from supercoiled pKL23-atp6-1-A, pKL23-trnM, or pKL23-trnM26. Labeled RNA products were separated in a 5% sequencing gel alongside an RNA size marker (M); sizes are given in nucleotides. Following transcript 5’ end mapping (Figure 3), major discrete RNA products (black arrowheads) were attributed to transcription initiation at Patp6-1-200 followed by termination at hisa (signals labeled Patp6-1-200-hisa) and thra (signal Patp6-1-200-thra) and initiation at PtmnM-98 or PtmnM26-893 followed by termination at hisa (signals PtmnM-98-hisa and PtmnM26-893-hisa). Additional minor signals (asterisk) may be due to differently migrating major products.

(B) Transcripts expected from initiation at mitochondrial promoters Patp6-1-156, Patp6-1-200, PtmnM-98, or PtmnM26-893 (bent arrows) and termination at hisa or thra (arrowheads) are indicated by horizontal black arrows labeled with the respective RNA lengths. Annealing sites of primers P2hisa (2) and P3hisa (3) employed for transcript 5’ end mapping are marked. nt, nucleotides.
or linearized templates. Enzyme preparations enriched in heter- 
drial and plastidial promoter sequences located on supercoiled 
and RpoTmp to initiate transcription in vitro at diverse mitochon-

A series of experiments compared the abilities of RpoTm, RpoTp, 
Selective Recognition of Mitochondrial Promoters by 
RpoTm, RpoTmp, and RpoTp in Vitro

Table 1. Promoter Recognition by RpoTm, RpoTmp, and RpoTp in Vitro

<table>
<thead>
<tr>
<th>Gene</th>
<th>Promoter</th>
<th>Sequence (5′−3′)</th>
<th>RpoTm</th>
<th>RpoTmp</th>
<th>RpoTp</th>
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<tr>
<td>mt18</td>
<td>Prm18-156</td>
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<td>–</td>
<td>+</td>
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<tr>
<td>atp6-1</td>
<td>Patp6-1-200</td>
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<td>–</td>
<td>–</td>
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<tr>
<td>atp9</td>
<td>Patp9-295</td>
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<td>–</td>
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<tr>
<td>cox2</td>
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<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>trnM</td>
<td>PtrnM-98</td>
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<td>rrn26</td>
<td>Prm26-893</td>
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<td>–</td>
<td>+</td>
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<td>–</td>
<td>ND</td>
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<td>–</td>
<td>–</td>
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<tr>
<td>atp6-1</td>
<td>Patp6-1-228/226</td>
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<td>–</td>
<td>+</td>
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<tr>
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<tr>
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<tr>
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<td>Prps3-1053</td>
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<td>–</td>
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<tr>
<td>cox2</td>
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<tr>
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<td>Patp6-2-507</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
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</tr>
<tr>
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<td>ND</td>
</tr>
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</table>

The preference for in vivo promoters over random initiation sites by RpoTm, RpoTmp, and RpoTp is indicated (+). In vivo start sites of transcription as determined previously (Kühn et al., 2005; Swiatecka-Hagenbruch et al., 2007), except Prm18-69 (Giese et al., 1996) and PcoxP-56 (Straman et al., 1998), are underlined in sequences; uppercase letters denote initiating nucleotides determined by 5′-RACE for transcripts synthesized in vitro by RpoTp (Pycf1-39) or RpoTm (all other promoters). Promoter cores are written bold, and the frequent mitochondrial promoter motif tatata(G) is in italics. ND, not determined.

a Stimulation of RpoTm-driven transcription by Patp8-157 was evident only from 5′-RACE performed on in vitro–synthesized transcripts.

b Initiation at Pcox2-345 was not observed in vivo and likely is an in vitro artifact.

Patp6-1-156 and Patp6-1-200 (Table 1). In vitro transcription from pKL23-trnM and pKL23-rm26 gave rise to specific RNA products (Figure 2A).

Through subsequent 5′-RACE, the 5′ termini of the discrete transcripts synthesized in vitro from pKL23-atp6-1-A, pKL23-

trnM, and pKL23-rm26 were found to accurately reflect those of transcripts generated in vivo from Patp6-1-200, Prm26-893, and Ptnm-98, respectively (Figure 3, Table 1; cf. Kühn et al., 2005), showing that recombinant RpoTm is able to correctly recognize and initiate transcription from different mitochondrial promoters in vitro. However, initiation at Prm26-893 and PtnM-98 was less efficient than at Patp6-1-200.

Selective Recognition of Mitochondrial Promoters by 
RpoT RNAPs in Vitro

A series of experiments compared the abilities of RpoTm, RpoTp, and RpoTmp to initiate transcription in vitro at diverse mitochondrial and plastidial promoter sequences located on supercoiled or linearized templates. Enzyme preparations enriched in hetero-

ologically expressed RpoT RNAPs (Figure 1) were used in these assays. Table 1 lists promoter sequences that were included in the study and indicates their in vitro use, which is illustrated in Figures 4 to 6 for selected promoters. Transcription initiation at organellar promoters in vitro was found to require supercoiled plasmid templates. Exclusively nonspecific transcription of linear templates by recombinant RpoT enzymes was observed in all in vitro assays performed in the course of this study (see lanes labeled “lin” in Figures 4A and 6A). Modified experimental conditions, such as altered concentrations of monovalent ions or the catalytic Mg2+, did not enable the enzymes to start RNA synthesis at promoters located on linear templates or to use promoters not recognized under the conditions of the standard in vitro transcription protocol, such as Patp9-652, Prps3-1053, Prps3-1133, and the A/T-rich PtnM-574/573, lacking apparent mitochondrial consensus promoter sequence elements (assays not displayed; see Table 1 for promoter sequences).

Of the two mitochondrial promoters present on pKL23-atp8-8, only Patp8-228/226 significantly supported specific transcription initiation by RpoTm, while Patp8-157, which displays a
sequence identical to Patp6-1-156 around the in vivo transcription start site, was apparently not recognized by RpoTm as a promoter (Figure 4A). The 5′-RACE performed on pKL23-atp6-1-A-derived RNAs confirmed the major defined transcripts to map to one out of two adenines in Patp8-228/226 used as initiating nucleotides by the mitochondrial transcription machinery in vivo (Figure 4C, Table 1). Extensive sequencing of cloned 5′-RACE products identified minor discrete transcript 5′ ends mapping to Patp8-157. However, these transcripts were not distinguishable from the background of nonspecific products in the autoradiograph.

RpoTm-driven transcription of pKL23-rrm18 was found to efficiently and accurately initiate at Prrn18-156 but not Prmn18-69 in vitro (Figure 4A). The minor signals indicated by grey arrows and marked with asterisks were assumed to correspond to differentially migrating major transcripts rather than additional defined RNA 5′ ends, as they did not give rise to discrete 5′-RACE products (Figure 4C). Minor signals at similar distances from major bands were likewise seen for the template pKL23-atp6-1 (Figure 2A). Transcripts with altered migration behavior may have retained unmelted secondary structures in the denaturing polyacrylamide gel.

To rule out the possibility that the failure of RpoTm to efficiently initiate at Patp6-1-156, Patp8-157, and Prmn18-69 was due to the presence of a second, favored upstream promoter on the plasmid template (see Figures 2B and 4B), additional templates were constructed containing the Patp6-1-156 or Prmn18-69 promoter region but lacking the upstream Patp6-1-200 or Prmn18-156 sequence, respectively. However, in vitro transcription of these templates did not initiate specifically at Patp6-1-156 or Prmn18-69 (data not shown).

Transcription of pKL23-cox2, which harbors two identified mitochondrial promoters, yielded discrete RNA products mapping to three different initiation sites (Figure 4). Through 5′-RACE, two of these sites were confirmed to correspond to the in vivo initiating nucleotides of Pcox2-210 and Pcox2-481. The Pcox2-481 sequence additionally gave rise to a transcript 5′ end not observed in vivo (Table 1). Erratic initiation by RpoTm occurred at a sequence that resembles Arabidopsis mitochondrial promoters but had not been detected to function as promoter in vivo. The 5′-terminal nucleotide of the nonspecific transcript was determined to correspond to position –345 upstream of the beginning of the Cox2 coding sequence on the Arabidopsis mitochondrial DNA, and the initiation site was denoted P^*cox2-345 (Figures 4A and 4C, Table 1). In assays using template pKL23-atp6-2, the RpoTm-derived RNA 5′ termini identified for Patp6-2-436 and Patp6-2-507 did not exactly correspond to the in vivo primary 5′ ends but mapped to positions one and three nucleotides, respectively, downstream of the correct start sites (Figure 4A, Table 1).

Minor discrete RNAs equal in size to those obtained with RpoTm were generated by RpoTmp from Patp6-1-916/913 (data not shown), Patp6-2-436, and Patp6-2-507 (Figure 4A). Precise mapping of the 5′ termini of these products was not performed, as they were hardly visible within the background of far more abundant nonspecific transcripts, thus rendering the identification of defined RNA 5′ termini extremely difficult. Synthesis of specific transcripts by RpoTmp was confined to these three promoters in the entire study (Figures 4A and 6A, Table 1), indicating fundamental differences between the abilities of RpoTm and RpoTmp to initiate transcription from mitochondrial promoters.

We also tested the ability of recombinant RpoTp to substitute for RpoTm in the in vitro transcription from mitochondrial promoters. Remarkably, discrete transcripts were made by RpoTp from a variety of mitochondrial promoter templates, which matched in size those made by RpoTm (Figure 4A, Table 1). Their precise 5′ termini were not determined.
of transcript lengths, it is likely that they were due to initiation at mitochondrial promoter sequences. Mitochondrial promoter recognition by RpoTp was not as efficient as by RpoTm, as judged from the ratio of specific and randomly initiated RNAs synthesized by these enzymes.

The RNAP preparations used in the experiments described above, though markedly enriched in RpoT RNAPs, contained several E. coli proteins (Figure 1), which might interfere with transcription or even be a precondition for the observed properties of the enzymes. We therefore tested whether a more

**Figure 4.** Distinct Transcriptional Properties of RpoTm, RpoTmp, and RpoTp.

(A) RpoTm (m), RpoTmp (mp), and RpoTp (p) were assayed for promoter-specific transcription from pKL23-atp6-2, pKL23-atp8-A, pKL23-rrn18, and pKL23-cox2 provided as supercoiled (ccc) or linearized DNA templates (lin). Owing to the high RNAP activity of RpoTmp (see Figure 1B), reactions set up with this enzyme contained only 1:20 of the [α-32P]-UTP added to assays with RpoTm and RpoTp. Transcripts were analyzed as in Figure 2A. Major discrete RNA products are indicated by arrows and, after transcript 5' end mapping (C), were attributed to transcription initiation at Patp6-2-436 followed by termination at hisa (signal P-436-hisa) and at Patp6-2-507 followed by termination at hisa (signal P-507-hisa), etc. Minor RpoTm-derived signals (asterisk) may be due to differently migrating major products. Signals denoted P*-345-hisa and P*-345-thra are due to nonspecific initiation from pKL23-cox2 (see text). Extended expositions of gels did not reveal additional bands.

(B) Transcripts expected from initiation at the introduced promoters followed by termination at hisa and thra or at the restriction sites EcoRI (E) used for linearization of pKL23-atp6-2 or XhoI (X) used for pKL23-atp8-A, pKL23-rrn18, and pKL23-cox2 linearization are indicated by horizontal black arrows labeled with the respective RNA lengths. Other symbols are as in Figure 2B.

(C) 5'-RACE was performed as detailed in Figure 3 on RNA molecules synthesized in vitro by RpoTm from supercoiled pKL23-atp6-2, pKL23-atp8-A, pKL23-rrn18, and pKL23-cox2 (chromatograms not displayed). Signals are labeled with the corresponding promoter name or initiation site.
purified preparation of RpoTm would still be able to correctly transcribe the template DNA. Additional purification steps led to RpoTm preparations that were virtually free of other proteins (Figure 5A). As shown in Figure 5B for Patp8-228/226, promoter recognition and transcription initiation by RpoTm in vitro remained unchanged over all steps of enzyme purification, indicating that contaminating E. coli proteins do not assist RpoTm in transcription initiation and supporting the idea that promoter recognition is an intrinsic property of RpoTm. RpoTmp, when purified to near homogeneity over a heparin column, was still unable to accurately initiate transcription from organellar promoters (data not shown).

Selective Recognition of Plastidial Promoters by RpoT RNAPs in Vitro

We likewise performed in vitro assays examining transcription from Arabidopsis plastidial promoters. NEP promoters were selected based on in vivo transcription initiation sites determined for several plastidial genes in Arabidopsis (Swiatecka-Hagenbruch et al., 2007). In these assays, both supercoiled and linearized pKL23 derivatives carrying the class Ia NEP promoters PaccD-252 and Pycf1-104, the class Ib NEP promoters PproB-300, PaccD-172, and Pycf1-39, or the class II NEP promoter PcpBP-57 from Arabidopsis served as DNA templates (see Table 1 for promoter sequences and their recognition in vivo). From in vitro transcription experiments followed by the determination of 5' ends of in vitro–synthesized transcripts, Pycf1-39 appeared to be the only NEP promoter stimulating specific transcription initiation by RpoTp (Figure 6, Table 1). All other NEP promoters tested did not give rise to discrete RNAs and were thus not preferred by RpoTp over random initiation sites (data not shown). Surprisingly, while the mitochondrial enzyme RpoTm recognized Pycf1-39 in vitro (Figure 6A), no specifically initiated transcripts were made by RpoTmp from any of the studied NEP promoters (Table 1).

RpoTm and RpoTp Recognize a Conserved Mitochondrial Promoter Sequence Element

The above-described in vitro assays revealed an intrinsic specificity of RpoTm and RpoTp for transcription initiation from diverse promoter sequences. We therefore tested if a functional promoter sequence element seen in many plant mitochondrial promoters and similarly in plastidial NEP promoters (YRTA motif; Rapp et al., 1993; Binder et al., 1995; Liere and Maliga, 1999) is important for promoter recognition by RpoT polymerases in our assays. We substituted the CATA motif of the wild-type Patp8-228/226 promoter with the sequence CtgA (Figure 7B). Changing these positions in the plastidial rpoB gene promoter was shown to greatly affect promoter recognition in in vitro transcription experiments with plastid extracts from tobacco (Liere and Maliga, 1999). Transcription assays with mutant DNA templates showed drastically reduced levels of correctly initiated transcripts for both RpoTm and RpoTp (Figure 7A), thus confirming the importance of these residues for promoter recognition by the two RNAPs.

DISCUSSION

Higher plants possess small families of nuclear RpoT genes that are related to the T7 bacteriophage RNAP. The dicotyledonous plant Arabidopsis has three such genes, RpoTm, RpoTp, and RpoTmp, which code for organellar RNAPs. Although it is generally accepted that RpoT gene products represent catalytic subunits of organellar transcription machineries in plants, direct evidence has been lacking for RpoT enzymes being involved in the transcription of mitochondrial and plastidial genes in photosynthetic eukaryotes. Our study establishes the three Arabidopsis RpoT genes to encode transcriptionally active RNAPs that prefer supercoiled over linear DNA templates (Figure 1). To elucidate functional differences between the enzymes, we have reconstituted the promoter-specific transcription of organellar genes in vitro from recombinant Arabidopsis phage-type RNAPs.

Intrinsic Promoter Specificities of RpoTm and RpoTp

In vitro transcription studies examined the abilities of the mitochondrial RNAP RpoTm, the plastidial RpoTp, and the
dual-targeted RpoTmp to transcribe DNA from mitochondrial and plastidial promoters located on supercoiled or linearized plasmid templates. No specific initiation of transcription was obtained with linear templates regardless of the RNAP tested. In this respect, RpoTm and RpoTp differ from the T7 RNAP, which is known to specifically transcribe circular and linear DNA templates in vitro (Portugal and Rodrı́guez-Campos, 1996). While RpoTmp exhibited no significant promoter recognition, RpoTm and RpoTp surprisingly accurately initiated transcription at several mitochondrial promoters and one plastidial promoter from supercoiled templates, as deduced from 5’ ends of in vitro–synthesized discrete RNAs (Table 1); 5’ termini of transcripts made in vitro corresponded mostly to those of primary transcripts observed in vivo in Arabidopsis organelles. RpoTm and RpoTmp were purified to near homogeneity (Figure 5), and although we cannot fully rule out the possibility that traces of contaminating E. coli proteins assisted RpoTm and RpoTp in promoter recognition, the latter is highly unlikely. To our knowledge, no studies have been published so far that would provide evidence for promoter recognition by bacteriophage or phage-type RNAPs depending on E. coli proteins. Overexpression of the histone-like protein HU was found to stimulate T7 RNAP activity in E. coli, possibly by binding to and altering supercoiling of the DNA (Morales et al., 2002). However, such a nonspecific stimulation of RpoTm or RpoTp by E. coli proteins would not account for the specific recognition of organellar promoter sequences seen in our in vitro transcription studies. We propose the observed promoter sequence specificity to be an intrinsic and cofactor-independent feature of the RpoTm and RpoTp polypeptides.

**Selective Promoter Recognition by RpoTm and RpoTp**

Recombinant RpoTm and RpoTp initiated transcription in vitro from diverse but not all promoter sequences tested. Of the promoters examined, all sequences displaying a TATATA element were recognized by RpoTm in vitro. Similarly, all promoters with...
a CGTA core stimulated transcription recognition, although PrmM-98, which is the only tested promoter possessing a CGTA motif but not a TATATA sequence, appeared to support initiation less efficiently. Of the promoters displaying a CATAGAGAG nonanucleotide motif, only Prm26-893 gave rise to discernible, though not abundant, transcripts. Transcription assays and 5’ end mapping of in vitro-synthesized RNAs together provided no evidence for transcripts initiated specifically at Patp6-1-156, Patp9-239, or the similar Prm18-69, and a minor stimulation of transcription initiation by Patp8-157 was revealed only by the sensitive 5’-RACE technique, indicating that RpoTm does not significantly prefer these in vivo promoters over random start points in vitro. Since despite the high sequence similarity of these promoters to Prm26-893 transcription is detectably initiated only at Prm26-893, already minor deviations from the Prm26-893 sequence may impede recognition by RpoTm. Alternatively, sequences beyond the 25 nucleotides displayed in Table 1 may determine promoter strength in vitro. By point mutagenesis of the Patp8-228/226 promoter in the CATAG motif (Figure 7), which is a frequent element in plastidial NEP and mitochondrial promoters, we demonstrated the importance of these residues for promoter recognition by RpoTm and RpoTp, thereby underpinning the enzymes’ capacity to directly and specifically interact with organellar promoter sequences.

While transcripts initiated at promoters Patp6-1-916/913 and Patp9-487, which do not possess a CRTA sequence element, had 5’ termini identical to those of transcripts made from these promoters in vivo, initiation at Pcox2-481 occurred at the correct nucleotide and in addition at a nearby downstream site not used in vivo. Transcript 5’ ends generated from Patp6-2-436 and Patp6-2-507 mapped to positions closely downstream of the in vivo initiating nucleotides. This may be either due to an altered or diverse in vitro activity of RpoTm compared with the in vivo initiation event or caused by intrinsic problems of the 5’-RACE procedure at the ligation step. One major discrete transcript that had not been observed in vivo (Kühn et al., 2005) was initiated in vitro from the cox2 upstream sequence. The function of a mitochondrial DNA sequence in vivo but not in vivo has been observed previously in in vitro studies employing maize (Zea mays) mitochondrial extracts as a source of transcription activity (Lupold et al., 1999). Rapid processing of transcripts not detected in vivo might account for discrepancies between transcription initiation in vitro and in vivo, although some sequences activating transcription in vitro might not serve as promoters in the mitochondrion.

RpoTp, which represents a NEP activity transcribing plastid genes (Liere et al., 2004), was found to be more selective than RpoTm in promoter recognition. Of the Arabidopsis NEP promoters tested for in vitro use by recombinant RpoTp, only Pycf1-39 gave rise to a promoter-specific transcript (Table 1). The limited recognition of NEP promoters by RpoTp in vitro, which may reflect the absence of RpoTp cofactors in our assays, is contrasted by an intrinsic specificity of this enzyme for several mitochondrial promoters. This finding is unexpected, as in vitro and in vivo import experiments suggest RpoTp to strictly localize to plastids (Hedtke et al., 1997, 1999). Remarkably, Pycf1-39, which is the only NEP promoter activating RpoTp in vitro, resembles in sequence the mitochondrial promoter Patp8-228/226 that is also efficiently recognized by both RpoTm and RpoTp in vitro (Figures 4 and 7, Table 1).

RpoTm and RpoTp May Need Auxiliary Factors for Transcription in Vivo

This study establishes the mitochondrial phage-type RNAP encoded by the nuclear RpoTm gene to recognize mitochondrial promoters of diverse architecture in vitro. It thus provides a direct linkage in the plant kingdom between an RNAP activity initiating transcription at mitochondrial promoters and a nuclear gene encoding a mitochondrial phage-type RNAP. Our in vitro transcription studies show that at least for the promoters found here to stimulate initiation by RpoTm, promoter sequence specificity is conferred by the RpoTm core enzyme and does not require auxiliary protein factors. The yeast mitochondrial RNAP Rpo41 was recently shown to possess the intrinsic ability to correctly initiate transcription in vitro at mitochondrial promoters on supercoiled or premelted DNA templates in the absence of the accessory factor sc-mtTFB (Matsunaga and Jaehning, 2004b). Addition of the cofactor increased abortive relative to productive transcription from premelted templates, indicating that sc-mtTFB does not confer promoter specificity but may stabilize the open promoter complex and modulate escape of the RNAP from promoter sequences into productive RNA synthesis. In line with observations of an intrinsic promoter specificity of yeast Rpo41 is the accurate transcription initiation by RpoTm and RpoTp at diverse mitochondrial promoters and one plastidial promoter of the Arabidopsis organellar DNAs that is described here. RpoTm used various but not all in vivo mitochondrial promoters tested and differed in its transcriptional performance from complex plant mitochondrial extracts (Hanic-Joyce and Gray, 1991; Rapp and Stern, 1992; Binder et al., 1995) in that it did not specifically initiate transcription at promoters located on linear DNA templates. Considering that in vivo, plant mitochondrial genomes are predominantly maintained as linear subgenomic DNA molecules of different sizes (Bendich, 1993; Backert et al., 1997), the in vitro performance of RpoTm implies the participation of an auxiliary factor(s) in modulating the DNA structure or in template melting and open promoter complex formation in in vitro transcription experiments using mitochondrial extracts and in vivo. According to our data, the function of such a factor(s) is most probably compensated for in vitro by a supercoiled DNA conformation that may facilitate opening of the double helix at many but not all promoters. RpoTp initiated transcription at only one out of six plastidial NEP promoters examined in vitro, and hence, most probably similar requires transcriptional cofactors for open promoter complex formation and transcription initiation. Notably, supercoiled DNA as a prerequisite for promoter recognition, which is displayed by RpoTm in vitro, has been reported previously for a NEP activity extracted from tobacco plastids (Liere and Maliga, 1999).

Evolutionary Diversification of Transcriptional Properties of RpoTm, RpoTmp, and RpoTp

The transcriptional role and localization of RpoTmp are still a matter of debate (Kabeya and Sato, 2005; Azevedo et al., 2006).
orthologs have been identified in monocotyledonous and dicot-
feature (Emanuel et al., 2004; Azevedo et al., 2006).

as the lineage leading to RpoTmp has evolutionarily diverged
specific RNA products from plastid DNA templates regardless of
vitro (Table 1). Similarly, RpoTmp synthesized exclusively non-
exceeded by nonspecific template transcription. Moreover, nine
initiation by RpoTm and even RpoTp with much higher efficiency.

The data presented here on RpoTm-., RpoTp-., and RpoTm-
dependent RNA synthesis in vitro are in favor of different and complementing functions of RpoTm and RpoTmp in mitochondria and of RpoTp and RpoTmp in plastids. Promoter recognition by RpoTmp in vitro was confined to Patp6-1-916/913, Patp6-2-436, and Patp6-2-507, which nevertheless induced transcription initiation by RpoTm and even RpoTp with much higher efficiency.

RNA synthesis from these promoters by RpoTmp was markedly exceeded by nonspecific template transcription. Moreover, nine out of 12 promoters used by RpoTmp did not activate RpoTmp in vitro (Table 1). Similarly, RpoTmp synthesized exclusively nonspecific RNA products from plastid DNA templates regardless of which class of NEP promoter was present on the template. The lack of promoter specificity of RpoTmp is particularly remarkable, as the lineage leading to RpoTmp has evolutionarily diverged from RpoTm more recently than RpoTp that still possesses this feature (Emanuel et al., 2004; Azevedo et al., 2006). RpoTp orthologs have been identified in monocotyledonous and dicotyledonous angiosperms (Weih, 2004) but appear to be absent in the green alga Chlamydomonas reinhardtii (A. Weih, unpublished data) and in the moss Physcomitrella (Kabeya et al., 2002; Richter et al., 2002). Different RNA synthesis rates and promoter specificities may be related to distinct in vivo tasks of RpoTm and RpoTmp in mitochondria and RpoTp and RpoTmp in plastids. It is possible though that effects of the thioredoxin tag on RNAP activity varied between the enzymes despite their N-terminal tags being identical. Removal of the tag from RpoTm, RpoTp, and RpoTmp had no effect on their ability to recognize promoters in vitro (A.-V. Bohne, unpublished data). In light of the in vitro initiation at diverse organellar promoters by RpoTm and RpoTp, it is unlikely that the apparent lack of specificity of RpoTmp for these promoters is due only to the absence of specificity factors in the in vitro transcription assay that would otherwise enable RpoTmp to efficiently recognize identical promoters. It is possible though that protein cofactors enable RpoTmp to recognize other, distinct organellar promoters in vivo. Alternatively, RpoTmp may play a role that does not involve RNA synthesis from organellar promoters. It may be speculated, for example, that RpoTmp functions as a polymerase priming DNA replication in mitochondria and/or plastids and initiates RNA synthesis as at yet undefined, possibly random sites. The mitochondrial RNAP of both humans and yeast functions in the transcription of mitochondrial genes as well as in initiation of DNA replication (reviewed in Leciner and Foury, 2000).

In conclusion, our study demonstrates the promoter specificities of the three Arabidopsis phage-type RNAPs to have diverged in the course of angiosperm evolution. Like the T7 RNAP, RpoTm and RpoTp can act as single-polypeptide enzymes in vitro and recognize mitochondrial promoters and at least one plastidial promoter. RpoTmp, despite its having diverged from RpoTm more recently than RpoTp, has effectively lost this ability. The yeast mitochondrial RNAP has been proposed to possess a polypeptide region corresponding in structure, though not in sequence, to the T7 RNAP specificity loop involved in promoter binding (Matsunaga and Jaehning, 2004a). Corresponding structures might be formed by plant RpoT polypeptides and could contribute to an intrinsic ability of phage-type enzymes to preferentially initiate transcription at particular sequences in vitro. This study, while revealing intrinsic promoter specificities of the RpoTm and RpoTp polypeptides, supports that phage-type RNAPs depend on cofactors for transcription initiation in plant organelles. Our in vitro assay provides a tool to characterize such auxiliary proteins.

METHODS

Expression and Purification of RNAPs

Sequences encoding amino acids 43 to 976 of RpoTm (locus tag At1g68990), amino acids 105 to 1011 of RpoTmp (locus tag At5g15700), and amino acids 96 to 993 of RpoTp (At2g24120) were amplified from reverse-transcribed mRNA-enriched Arabidopsis thaliana RNA using primer pairs listed in Supplemental Table 1 online and ligated into pBAD/Thio-TOPO (Invitrogen) to yield plasmids pBAD/Thio-HisRpoTm, pBAD/Thio-HisRpoTmp, and pBAD/Thio-HisRpoTp, encoding thioredoxin-hexahistidine–tagged RpoTm, RpoTmp, and RpoTp. Recombinant RNAPs were overexpressed in Escherichia coli BL21 Codon Plus RIL (Stratagene), purified over Ni²⁺-NTA agarose (Qiagen), and stored in 20 mM Tris/HCl, pH 7.8, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 50% (v/v) glycerol at −20°C for 2 to 6 weeks. These enzyme preparations were used for most experiments.

For further purification of RpoTm, Ni²⁺-NTA agarose–purified proteins were passed over a heparin matrix (Amersham Pharmacia Biotech). Bound protein was washed with 0.3 M NaCl and 0.1 M Tris/HCl, pH 7.8, and eluted with 0.8 M NaCl and 0.1 M Tris/HCl, pH 7.8. Eluted proteins were subsequently resolved by gel filtration chromatography using a TSK gel 2000 SWXL column (TosoHaas) at a flow rate of 0.7 mL/min. Eluting buffer was 20 mM Tris/HCl, pH 7.8, 0.3 M NaCl, 0.5 mM EDTA, and 1 mM DTT. Samples recovered from all purification steps were stored as detailed above. For supplementary experiments, RpoTmp was further purified and subjected to heparin affinity chromatography following purification over Ni²⁺-NTA agarose. The enzyme was stored as described.

Proteins were analyzed by SDS-PAGE according to Laemmli (1970).

RNAP Activity Assay

RNAP activity was measured as incorporation of [α-32P]-UTP into transcripts synthesized in vitro using 1 μg of activated calf thymus DNA (inhibitor studies) or 0.5 μg supercoiled or EcoRI-linearized pkL23 as template. Reactions additionally contained 40 mM Tris/HCl, pH 7.9, 10 mM NaCl, 6 mM MgCl₂, 10 mM DTT, 2 mM spermidine, 500 μM each ATP, CTP, and GTP, 25 μM unlabeled UTP, and 1 μCi of [α-32P]-UTP (5000 Ci/ mmol), 10 units of RNase inhibitor (Fermentas), and 2 pmol recombinant

Transgenic Arabidopsis plants carrying a T-DNA insertion in the RpoTmp gene displayed no apparent effect on mitochondrial transcript accumulation (Baba et al., 2004). Based predominantly on the observation that in the mutant, the induction of several plastid genes in dark-grown seedlings upon illumination was delayed, Baba et al. (2004) proposed RpoTmp to be the key RNAP transcribing organellar genes during early seedling development and favored a role of both RpoTm and RpoTp at a later developmental stage. This suggested function is not supported by our previous study demonstrating that RpoTm and RpoTmp genes exhibit fully overlapping expression patterns in different tissues and at different developmental stages in Arabidopsis (Emanuel et al., 2006). From a phenotypical comparison of RpoTp-defective, RpoTmp-defective, and double mutant plants, Hricova et al. (2006) concluded RpoTp and RpoTmp to have redundant functions in plant development.

 Samples recovered from all purification steps were stored as detailed.
RNAP, 0.5 units of E. coli core RNAP (Epitcentre), or 4 units of T7 RNAP (Fermentas) in a final volume of 15 μL. In inhibitors tests, rifampicin (Roth; 0.1 or 1 μg/mL) or actinomycin D (Roth; 10 or 100 μg/mL) was added. RNAP assays were allowed to proceed for 30 min at 30°C. Two 5-μL aliquots per reaction were subsequently spotted onto DE81 DEAE cellulose filters (Whatman); unincorporated label was removed by washing filters 3× 15 min in 0.21 M Na2HPO4. Filters were then dried, and filter-bound labeled transcripts were quantified in an LS6000SC liquid scintillation counter (Beckman). RNAP activities were calculated as averages of three independent experiments.

**Preparation of in Vitro Transcription Templates**

Mitochondrial and chloroplast DNA fragments were PCR-amplified from total Arabidopsis DNA with primer pairs listed in Supplemental Table 2A online. PCR products were SacI/PstI-digested and ligated into SacI/PstI-cleaved pKL23 (Liere and Maliga, 1999) upstream of terminator sequences to produce plasmid templates for transcription assays. Cloned templates were purified from E. coli using theQiagen Plasmid Midi kit. Linearized templates were generated by an Xhol or EcoRI digest and purified via QiAQuick spin columns (Qiagen). In vitro transcription templates with their encoded promoters and oligonucleotides employed for plasmid construction are listed in Supplemental Table 2A online.

For the construction of DNA templates used for point mutational analysis of Patp8-228/226, each oligonucleotide pair (listed in Supplemental Table 2B online) was incubated for 10 min at 85°C and cooled down slowly. Annealing products were used directly for ligation into SacI/EcoRI-cleaved pKL23.

**In Vitro Transcription Assay**

Standard assays were performed for 45 min at 30°C and essentially followed the protocol of Falkenberg et al. (2002). Reactions contained 6.7 mM Tris/HCl, pH 7.9, 6.7 mM KCl, 6.7 mM MgCl2, 0.67 mM DTT, 0.067% (w/v) BSA, 267 μM each ATP, CTP, and GTP, 13 μM unlabeled UTP, and 10 μCi of [α-32P]-UTP (3000 Ci/mmol), 24 units of RNase inhibitor, and 200 ng of template DNA in a final volume of 15 μL. Reactions were started by adding 400 fmol of recombinant RpoTM, RpoTmp, or RpoTp and stopped with 115 μL of RNA extraction buffer (6 M urea, 360 mM NaCl, 20 mM EDTA, 10 mM Tris/HCl, pH 8.0, and 1% [w/v] SDS) and 20 μL of 2.25 M sodium acetate, pH 5.2. Nucleic acids were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated from the aqueous phase, and washed with 70% (v/v) ethanol, and ethanol, and washed with 70% (v/v) ethanol.

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**Accession Numbers**

Arabidopsis Genome Initiative locus identifiers for the phage-type RNAs RpoTM, RpoTmp, and RpoTp are At1g68990, At5g15700, and At2g24120, respectively.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Table 1.** Primers Used for the Construction of Protein Expression Vectors.

**Supplemental Table 2.** Primers Used for the Construction of in Vitro Transcription Templates and Oligonucleotides Used for Construction of DNA Templates for Point Mutational Analysis of Patp8-228/226.

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