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Separation of two classes of plastid DNA-dependent RNA polymerases that are differentially expressed in mustard (*Sinapis alba* L.) seedlings

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Abstract

Chloroplast and etioplast *in vitro* transcription systems from mustard have different functional properties, which is reflected in differences in phosphorylation status. Here we report another transcription control mechanism, which involves two plastid DNA-dependent RNA polymerases designated as peak A and peak B enzymes. Both are large multi-subunit complexes, but differ in their native molecular mass (> 700 kDa for peak A and ca. 420 kDa for peak B) and in their polypeptide composition. The A enzyme is composed of at least 13 polypeptides, while the B enzyme contains only four putative subunits. Peak B activity is inhibited by rifampicin, whereas that of peak A is resistant. RNA polymerase activity was compared for plastids from cotyledons of 4-day-old seedlings that were grown either under continuous light (chloroplasts) or in darkness (etioplasts), or were first dark-grown and then transferred to light for 16 h ('intermediate-type' plastids). While the total activity was approximately the same in all three cases, enzyme B was the predominant activity obtained from etioplasts and enzyme A that obtained from chloroplasts. Both had equal activity in preparations from the 'intermediate-type' plastid form. Both activation/inactivation and differential gene expression seem to play a role in the regulation of the plastid transcription machinery.

Introduction

Plastids and mitochondria contain their own DNA and a complete gene expression system, but they nevertheless depend on nuclear gene products that are imported into these organelles [4, 5, 19]. Nucleo-plastidic interactions not only play an important role in the assembly and function of major plastid proteins such as Rubisco and the photosynthetic complexes, but also seem to control the organelle gene expression system itself [3, 59, 52].

Since the initial findings on RNA synthesis in isolated chloroplasts [29] the organelle transcription apparatus has been extensively studied (for review see [3, 26, 40]). One major approach towards the understanding of the basic mechanisms and cellular integration of plastid gene

This paper is dedicated to Professor Achim Trebst on the occasion of his 65th birthday.

transcription was the purification of a soluble, DNA-dependent chloroplast RNA polymerase from maize [7, 58, 28]. Using different purification schemes, similar preparations were also obtained from other species such as spinach [36, 35, 34] and pea [61, 49]. However, the results of this extensive biochemical work have not yet led to a common definition of subunit structure and function of chloroplast RNA polymerase(s).

Important clues came from the molecular dissection of the plastid DNA using recombinant techniques. It was shown for several plant species that their organelle genome contains homologues of the *rpoA*, *rpoB* and *rpoC* genes from *Escherichia coli*, which encode the α, β and β' subunits, respectively, of the bacterial RNA polymerase [24, 47, 56, 57, 67, 16]. The *in vivo* function of these genes could be demonstrated [24, 54, 48] and, in the case of maize, the gene products were obtained as subunits of purified plastid RNA polymerase and were identified by N-terminal sequencing [22, 23].

Although the existence of an '*E. coli*-like' RNA polymerase in plastids is now widely accepted, increasing evidence suggests that this enzyme might not be exclusively responsible for plastid RNA synthesis. For example, the complex polypeptide pattern of the purified preparations of chloroplast RNA polymerase from spinach [34] and pea [49] could not be fully reconciled with the expected subunit structure inferred from gene analysis. Likewise, the exact relationship among the (soluble) '*E. coli*-like' enzyme and a DNA-associated activity (TAC = transcriptionally active chromosome) [17, 38] still remains to be clarified. Initial indications for the existence of more than one type of plastid RNA polymerases were obtained by studies with the antibiotic rifampicin, a potent inhibitor of bacterial RNA polymerase [18], which was found to partially inhibit crude RNA polymerase preparations from *Chlamydomonas* [60] and maize [6]. However, subsequent work using more highly purified plastid enzymes did not show rifampicin sensitivity (reviewed in [26]).

More recent indications for a second, possibly nuclear-coded RNA polymerase, come from *in vivo* experiments with three different plant systems, i.e. heat-bleached rye seedlings [11], the 'albostrians' mutant of barley [21], and the parasitic plant *Epifagus* [13, 43]. Although each of these systems contains defective plastids, either by loss of functional organelle ribosomes (rye and barley) or by lacking certain plastid genes including the rpoBC_1C_2 operon (*Epifagus*), plastid RNA sequences were detected *in vivo* in all three cases. Hence, these findings could be easily reconciled with the existence of a second (nuclear-encoded) RNA polymerase inside the plastid compartment, although it is difficult to eliminate the possibility that the RNA detected in these *in vivo* experiments might come from a minor fraction of cells that remained fully functional. This emphasizes the need for direct biochemical evidence for the proposed nuclear-encoded plastid RNA polymerase.

We have been interested in transcriptional control operating during plastid development in the crucifer mustard (*Sinapis alba*). Previous studies have shown differential *in vivo* transcript patterns for plastid genes from light-grown versus dark-grown seedlings [39, 25, 46]. Further work suggests that this is reflected in part in changes in properties of the organelle transcription apparatus [51, 10, 15, 65, 63]. In the present study, we purified RNA polymerase activities from three different plastid types, i.e. chloroplasts, etioplasts, and partially converted 'intermediate-type' plastids from greening seedlings. In each case, two different enzyme forms, designated peak A and peak B, were detected during purification. We show that peak B resembles the bacterial enzyme, while peak A does not. We describe enzymatic characteristics, native molecular weights and polypeptide patterns of the A and B activities, and compare their relative activities in preparations from the three different plastid types.

Materials and methods

Preparation of plastid RNA polymerase activities

4-day-old mustard seedlings were grown either 96 h in the dark, 80 h in the dark followed by 16 h

illumination, or 96 h in the light, and plastids were isolated as described [7, 51]. Etioplasts were found to be free of mitochondrial contamination as suggested by the lack of oxygen consumption and absence of fumarase activity [37]. The plastids were lysed and transcriptionally active material then purified by chromatography on a 8 ml heparin-Sepharose CL-6B column [9, 15, 65, 63]. The transcriptionally active fractions eluting at 0.1–0.9 M $(NH_4)_2SO_4$ were pooled and then dialysed against buffer containing 50 mM Tris-HCl pH 7.6, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol, 50 μg/ml PMSF, 1 mM benzamidine and 0.1% Triton X100. 400 μl of this solution was layered on top of a 3.6 ml linear glycerol gradient containing 15–30% glycerol, 50 mM Tris-HCl pH 7.6, 0.1 mM EDTA, 1 mM 2-mercaptoethanol and 0.1% Triton X100 and centrifuged at 32000 rpm (120000 × *g*) for 15 h in a Kontron TST 60.4 rotor. After centrifugation the glycerol gradients were fractionated into 200 μl aliquots. These fractions were immediately analysed.

RNA polymerase assay

In vitro transcription activity was assayed in a standard 50 μl reaction mixture containing 50 mM Tris-HCl pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 0.6 mM each of ATP, CTP and GTP, 12.5 μM [5,6-^{3}H]UTP (1 Ci/mmol; Amersham), 15 μl protein sample and 5 μg heat-denatured calf thymus DNA as template. After incubation for 30 min at 30 °C, the total amount of RNA synthesized was determined by scintillation counting of trichloroacetic acid (TCA)-precipitable material [51].

Denaturing SDS-PAGE and protein staining

Polypeptides were analysed by denaturing SDS-PAGE on 5–15% polyacrylamide gels [32], followed by silver staining [42]. The sample buffer contained 4% SDS, 2% bromophenol blue, 2% glycerol and 75 mM Tris-HCl pH 6.8 as well as 10 mM each of PMSF, benzamidine, phenanthroline and ε-amino-n-caproic acid. This non-reducing sample buffer was found to increase the quality of the resolution of protein bands without artefactual bands that were sometimes generated in the presence of reducing agents. For protein denaturation, samples were then incubated at 30 °C for 30 min prior to electrophoresis. Higher temperatures resulted in protein degradation. For two-dimensional gel electrophoresis the same sample buffer, containing in addition 2% 2-mercaptoethanol, was used.

Protein quantification

Protein concentration of samples was determined by the Bradford assay [8].

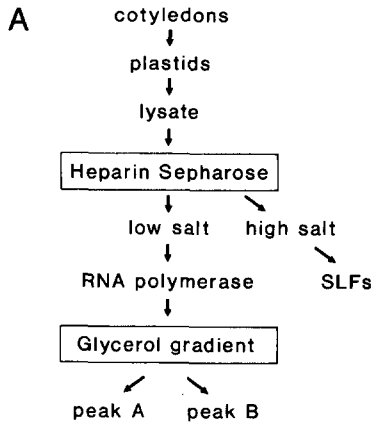
DNA polymerase activity assay

DNA polymerase activity in glycerol gradients was determined in a standard reaction mixture containing 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl, 0.1 mM each of dGTP, dCTP and dTTP, 0.01 mM of dATP, 0.01 mM of [^{32}P]-dATP (400 μCi/mmol, Amersham), 20 μl protein samples and 5 μg single-stranded plasmid pBS (Stratagene) annealed with T7 primer as template. After incubation for 30 min at 30 °C, the total amount of DNA synthesized was determined by scintillation counting of TCA-precipitable material.

Results

Two different RNA polymerase activities from plastids of mustard seedlings

The initial goal of our work was to develop a rapid and efficient purification procedure for chloroplast RNA polymerase that would allow further functional characterization of the enzyme. As shown in the purification scheme in Fig. 1A, the procedure involved chromatography of the



B

A/B activity ratio	
ET	1 : 7,4
PT16	1 : 1
CP	8,9 : 1

Fig. 1. A. Purification scheme for plastid RNA polymerase activities from mustard (*Sinapis alba* L.). Plastids prepared from cotyledons of 4-day-old seedlings were lysed and the proteins then loaded on a heparin-Sepharose column. Bound proteins were eluted with a linear ammonium sulphate gradient. The high-salt material (1.0–1.3 M) lacks transcription activity and contains free 'sigma-like' transcription factors (SLFs) [65]. The low-salt fractions (0.1–0.9 M) containing RNA polymerase activity were pooled and further purified by glycerol gradient centrifugation. The analysis of the gradient fractions showed two distinct RNA polymerase activities designated as peak A and peak B. B. Relative activities of peak A and peak B in different plastid types. Plastids were isolated from seedlings that were either dark-grown (ET, etioplast), dark-grown and then illuminated for 16 hours (PT16, plastids after 16 h illumination), or light-grown (CP, chloroplasts). The values for the A/B activity ratios were obtained by integrating the peaks shown in Fig. 2 (see also Table 1).

chloroplast lysate on heparin-Sepharose as described [65, 63, 64], followed by an additional glycerol gradient centrifugation step. This simple two-step procedure yielded reasonable amounts of enzyme that were free of contaminating lower-molecular-weight proteins but also resulted in an unexpected separation of two RNA polymerase activities designated peak A and peak B (Fig. 1). These two peaks appear to represent different RNA polymerase forms of distinct size and polypeptide composition (Figs. 2, 3 and 4).

When the purification procedure outlined in Fig. 1A for chloroplasts (CP) was repeated with etioplasts (ET) or 'intermediate'-type plastids from greening seedlings (PT16), in each case both peak A and peak B could be detected after the glycerol gradient step (see Figs. 2A and 2B for CP, Figs. 2C and 2D for ET and Figs. 2E and 2F for PT16), yet with different relative activities (Fig. 1B and Table 1).

As shown in Table 1, using the same amount of starting material, there was a weak increase in total RNA polymerase activity after the heparin-Sepharose step from etioplasts via PT16 plastids to chloroplasts, whereas the specific activity decreases in the reverse order. Integration of the peak A and peak B activities after the glycerol gradient step (Figs. 1B and 2, Table 1) revealed an activity shift from peak B in etioplasts towards peak A in chloroplasts (almost one order of magnitude variation in A/B ratio). The PT16 plastids showed an intermediate situation with almost equal peak A and B activities.

Transcriptional properties of the peak A and peak B RNA polymerase forms

The two resolved peaks were characterized by variation of the standard *in vitro* transcription assay (see 'Materials and methods'). As shown in Table 2, both RNA polymerase activities are DNA-dependent. In both cases, transcription takes place with either denatured or supercoiled DNA templates and is inhibited by actinomycin D. Omission of one or three of the four ribonucleotides resulted in a total loss of activity. The ribonucleotides cannot effectively be replaced with deoxyribonucleotides, although a significant residual activity was noted in the case of the B enzyme. However, this is probably not an inherent property of this enzyme form but may be due to contamination. A DNA polymerase assay (see 'Materials and methods') detected activity in the upper portion of the gradient, with a maximum only three fractions above that of peak B (data not shown).

Inhibition experiments showed that both

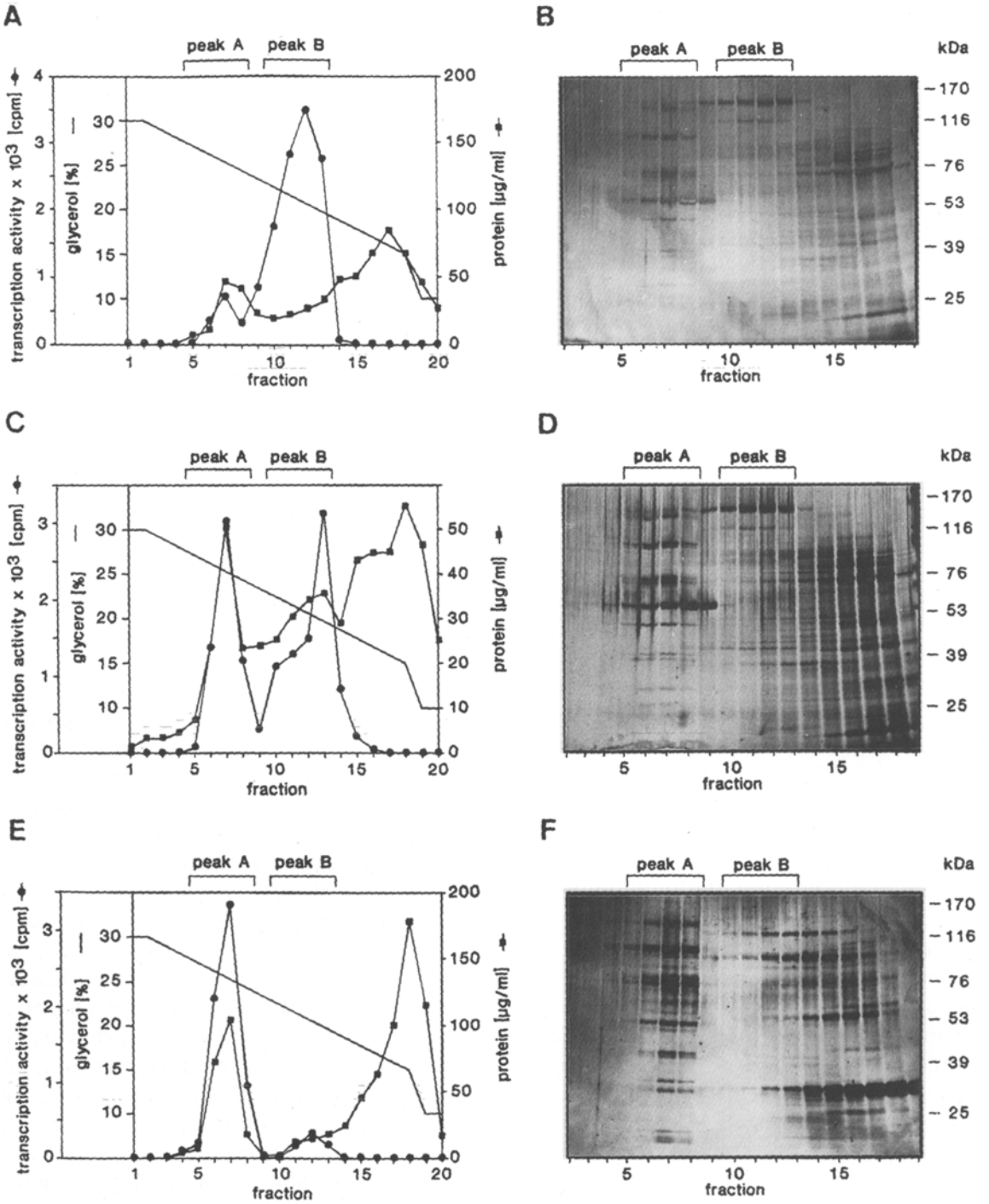


Fig. 2. Separation of RNA polymerase activities by glycerol gradient centrifugation. A, C, E. Protein content and activity profiles along the gradients. B, D, F. Silver-stained polypeptides of fractions from the gradients shown in A, C and E. RNA polymerase peak A and peak B regions are indicated on top of each panel. Proteins were prepared from etioplasts (A, B), plastids after 16 h illumination (C, D), and from chloroplasts (E, F). The heavy band at 55 kDa in panels B and D represents Rubisco large subunit which is associated with heparin-Sepharose-stage RNA polymerase preparations to variable degrees (not shown). In E and F, a chloroplast preparation was chosen which contains little contamination by this band. For sizes of marker proteins (right margins of panels B, D and F), see legend to Fig. 4.

Table 1. Purification of mustard peak A and peak B RNA polymerase activities from different plastid types

Purification step	Plastid type[a]	Total protein[b] (mg)	Total activity[c] (cpm × 10^3)	Yield (%)	Specific activity (cpm × mg^{-1})	Purification factor[d] (fold)
heparin-Sepharose:						
	ET	6.57	2654	100	403.9	1.00
	PT16	11.07	2716	100	245.3	1.00
	CP	24.89	2851	100	114.5	1.00
glycerol gradient:						
	ET	1.48	302	11.04	204.1	0.51
peak A	PT16	1.57	1324	48.70	843.3	3.44
	CP	4.18	2306	80.90	551.7	4.80
	ET	3.98	2242	84.50	563.3	1.39
peak B	PT16	4.17	1340	49.30	321.3	1.31
	CP	1.47	251	8.80	170.7	1.49

[a] Abbreviations: ET, etioplast; PT16, 'intermediate' plastid type after 16 h illumination; CP, chloroplast.
[b] From 1.5 kg mustard cotyledons as starting material.
[c] Data presented are mean values obtained by three independent determinations. Background activities determined with samples that were TCA-precipitated at zero times (less than 10% of values for active samples) were previously subtracted.
[d] No activity was detected prior to heparin-Sepharose. Based on comparison of polypeptides, the latter fraction represents a > 500-fold purification over the plastid crude extract.

peak A and peak B are heparin-sensitive. No effect was noted in the presence of relatively high concentrations of α-amanitin, indicating that there was no contamination by nuclear RNA polymerases. Using rifampicin, an inhibitor of prokaryotic RNA polymerases [18], showed that peak A was unaffected or even slightly stimulated in the presence of this drug. In contrast, an almost complete inhibition of the peak B activity resulted at inhibitor concentrations as low as 0.1 μg/ml. This inhibitory effect was found regardless of whether denatured or supercoiled DNA templates were used. Another rifamide, rifamycin SV, resulted in a comparable degree of inhibition of peak B activity (Table 2).

A partial inhibition by rifampicin was noted for the transcriptionally active (low-salt) fraction upon heparin-Sepharose chromatography, i.e. the first purification step where RNA polymerase activity became detectable (Table 1). Using 10 μg/ml of the drug, 78.9% (ET), 51.3% (PT16), and 20.2% (CP) inhibition was observed. These values correspond to enzyme A/B ratios of 1:3.7 (ET), 1.1:1 (PT16), and 3.9:1 (CP), respectively, which is within the range of values obtained for the separated enzyme forms after glycerol gradient centrifugation (Table 1).

Molecular weight and polypeptide composition of peak A and peak B enzyme forms

To estimate the molecular weight of the peak A and peak B activities, we used calibrated glycerol gradients containing marker proteins. As shown in Fig. 3, this gave native molecular weight values of around 420 kDa for peak B and of at least 700 kDa for peak A. Due to the lack of appropriate high-molecular-weight marker proteins the latter value is a minimum estimate. Peak A showed a complex polypeptide pattern, consisting of seven major bands (141, 110, 107, 78, 72, 48 and 29 kDa) and at least six minor bands (72, 36, 35, 26, 16 and 13 kDa). The sum of these molecular masses is 781 kDa, which is in reasonable agreement with the molecular mass of the native complex. Further evidence that these 13 polypeptides are constituents of the peak A enzyme was obtained by two-dimensional analysis using non-denaturing gel electrophoresis in the first dimension and SDS-PAGE in the second dimension (data not shown). This indicates that the peak A enzyme is a large multi-subunit complex, which is not a larger aggregate of the peak B enzyme, but rather contains a distinct polypeptide pattern.

Table 2. Characterization of purified plastid RNA polymerase.

Reaction mixture	RNA polymerase activity (%)		
	Peak A	Peak B	*E. coli*
Control (full system)	100.0	100.0	100.0
– template DNA	2.3	0	0
+ Actinomycin D			
10 μg/ml	29.9	5.4	37.3
100 μg/ml	2.8	4.7	18.8
– ATP	0	0	2.9
– ATP, GTP, CTP	0	0	0
+ dNTPs	0	14.3	2.5
supercoiled template DNA	48.8	68.8	n.d.
+ Heparin			
0.1 μg/ml	0	0	1.2
1 μg/ml	0	0	1.0
+ α-Amanitin			
100 μg/ml	95.9	117.9	110.5
250 μg/ml	110.5	123.3	118.0
+ Rifampicin			
0.01 μg/ml	115.5	64.3	61.6
0.1 μg/ml	114.8	10.0	2.4
1 μg/ml	117.4	5.5 (10.7)*	1.1
+ Rifamycin SV			
0.01 μg/ml	n.d.	95.9	n.d.
0.1 μg/ml	n.d.	28.1	n.d.
1 μg/ml	n.d.	11.3	n.d.

Transcription assay using either *E. coli* RNA polymerase or glycerol gradient purified plastid enzyme peak A or B were carried out as described in Material and methods in the absence or presence of the indicated assay components or inhibitors. To test deoxyribonucleotides (dNTPs) as substrates for peak A and B, DNA polymerase assays were performed (see Materials and methods), with T7 DNA polymerase as a positive control (data not shown). Stock solutions of inhibitors were 1 mg/ml each. Actinomycin D, heparin and α-amanitin were dissolved in water and the rifamides in methanol. Control reactions each contained the same amounts of stock solution without inhibitors.

* Numbers in parenthesis: percentage of activity with supercoiled template: plasmid pSA05/H120 carrying the plastid *psbA* promoter in pUC13 [15].

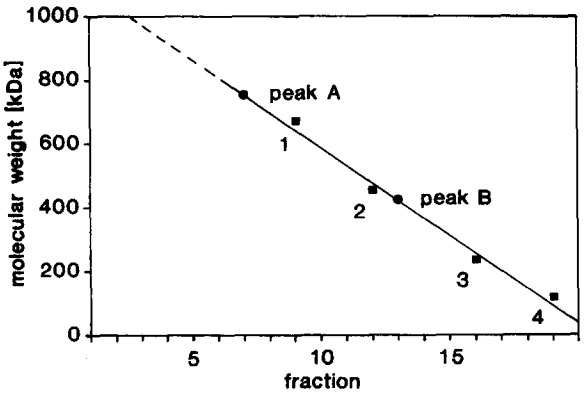


Fig. 3. Molecular weight assessment of peak A and peak B RNA polymerase in glycerol gradients. The gradients were calibrated with native marker proteins, which are indicated by numbers (■). 1, thyreoglobuline (669 kDa); 2, *E. coli* RNA polymerase (450 kDa); 3, β-galactosidase dimer (232 kDa); 4, β-galactosidase monomer (116 kDa). The peak positions of the markers were determined by fractionation of the gradient, followed by SDS-gel electrophoresis and silver staining. Gradient-purified peak A and peak B were layered on top of the same gradients as the markers and their positions were determined using the RNA polymerase assay (●).

The analysis of the polypeptide composition of the peak B activity proved to be more difficult because of contaminating proteins from the upper portion of the glycerol gradient (see Fig. 2). The combined kDa values of all consistently observed polypeptides (dots in lane 'B' in Fig. 4) gave a total molecular mass of about 700 kDa, not corresponding to the 420 kDa native peak B complex (Fig. 3). Unfortunately, two-dimensional gel electrophoresis could not be used as an alternative method because of instability of the peak B enzyme in the native first dimension (not shown). Nevertheless, the three most prominent polypeptides associated with peak B fractions in Figs. 2B and 2D, with apparent molecular masses of 154, 120 and 38 kDa (arrowheads in Fig. 4, lane 'B'), co-purify with the enzymatic activity (Fig. 2), while other bands seem to represent contaminants from upper fractions of the glycerol gradient. The apparent sizes of these three major peak B-associated polypeptides closely match those of the *rpo* gene products β″, β and α from spinach [24]. In addition, a less intensely stained 72 kDa polypeptide in Fig. 4, lane 'B', could be the mature processed form of the β′ subunit, which has a molecular mass of 71 kDa in spinach [14]. These four polypeptides together account for 384 kDa, which compares reasonably with the

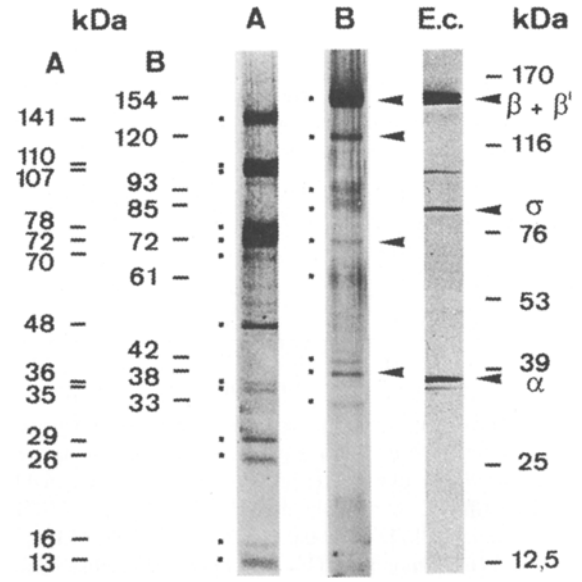


Fig. 4. Molecular sizes of putative subunits of the two plastid RNA polymerase forms. Polypeptide patterns are shown for peak A from chloroplasts (lane 'A'), peak B from etioplasts (lane 'B'), and for *E. coli* RNA polymerase (lane 'E.c.'; β', β, σ and α polypeptides marked by arrows). The kDa values for peak A and B polypeptides (dots in lanes 'A' and 'B') are given in the left margin. Several unlabelled minor bands in lane 'A' were not detected after two-dimensional gel electrophoresis. Arrows to the right of lane 'B' mark the putative plastid RNA polymerase subunits β'' (154 kDa), β' (120 kDa), β (71–78 kDa), and α (38 kDa). Other polypeptides of peak B are not considered to be subunits as they are enriched in the top portion of the glycerol gradient (see Fig. 2). The marker proteins with sizes indicated in the right margin were α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) glutamic dehydrogenase (53 kDa), aldolase (39 kDa), chymotrypsin (25 kDa), and cytochrome C (12.5 kDa).

420 kDa for the native peak B enzyme (Fig. 3), considering an additional 29, 52 or 67 kDa for one of the three known sigma-like factors (SLFs) present in low amounts [65, 63].

In the absence of amino acid sequence information and comparison to the mustard *rpo* genes, this is a tentative assignment of putative subunits. It could be argued that, for example, the 72 kDa (β') band is quite weak in Fig. 4, lane B, and also in the peak B fractions of the glycerol gradients in Figs. 2B and 2D, while its intensity seems to increase towards the top of the gradients. However, although partial dissociation of the peak B holoenzyme cannot be excluded, the strong 70–75 kDa band close to the top of the glycerol gradients clearly consists of several different polypeptides that can be separated upon prolonged electrophoresis (not shown).

Discussion

A number of different purification schemes have been developed to purify plastid RNA polymerase (for review see [3, 26]). We present here a short two-step procedure designed to minimize loss of enzymatic activity and to reduce as much as possible the dissociation of subunits or additional factors during the purification. Furthermore, this short and reproducible technique has been found suitable for comparative investigations of RNA polymerase activities from different plastid types.

The accumulation of some but not all plastid mRNAs was reported to be light-stimulated in a number of plant species, including maize [2, 53], mustard [39, 14, 25], pea [62, 66], spinach [12, 45] and sorghum [55]. It has become a common notion that the degree of light regulation of plastid gene expression seems to depend on the species, developmental stage and tissue type (see e.g. [41, 20]). Since the molecular mechanisms involved appear partially to reside at the transcriptional level [30, 15, 64], we were interested to characterize the organelle transcription apparatus in greater detail, using different plastid types.

We have purified RNA polymerase activity from chloroplasts of light-grown seedlings and from etioplasts of dark-grown seedlings. In addition, we used 'intermediate-type' plastids from seedlings that were first grown in darkness and then transferred to light. As shown in Table 1, the total activity obtained from equal amounts of starting material (1.5 kg of mustard cotyledons) was almost constant or slightly increased from etioplasts towards chloroplasts, whereas the specific activity of the preparations decreased in the opposite direction. This most likely reflects the accumulation of photosynthesis-related major proteins during light-induced greening.

Further purification of the RNA polymerase

activity from each plastid type by glycerol gradient centrifugation led to separation into two activity peaks, designated peak A and peak B, which appeared to be inversely regulated in these two plastid types. While there is an almost tenfold excess of peak B activity over that of peak A in etioplast preparations, the reverse is true for the chloroplast preparations, and both peaks have approximately the same activity in preparations obtained from the 'intermediate-type' plastids of greening seedlings (Table 1). Interestingly, the protein content of the corresponding peaks from etioplasts and intermediate plastids is very similar, but is different from that of the respective chloroplast peak (Table 1). This might mean that two mechanisms contribute to the light-dependent differentiation of the plastid transcription apparatus during greening. Temporally the first event seems to be an activation of the 'A' enzyme and inactivation of the 'B' enzyme, possibly by protein modification such as phosphorylation [64]. This is then followed by a subsequent increase (peak A) and decrease (peak B) in the actual concentration of proteins associated with the two activity peaks. In essence, these findings indicate a significant change in the plastid transcription apparatus during the light-induced conversion from the etioplast towards the chloroplast state. This change seems to consist of two phases, the first of which is characterized by altered activities of peak A versus peak B in the absence of significant changes in protein content. The second phase appears to consist of differential gene expression for individual components of the transcription machinery, reminiscent of those known for other macromolecular complexes of the organelle such as the photosynthetic apparatus [44, 20].

What might be the relationship and function of the peak A and peak B activities? Both peaks show a distinct migration behaviour in glycerol gradients indicating different size of the active complexes (Fig. 3). Although these experiments leave the formal possibility that peak A might represent a larger aggregate (e.g. dimer) of the peak B enzyme form, this is unlikely in view of the distinct polypeptide patterns obtained upon SDS-PAGE (Fig. 4). Virtually none of the polypeptides in each preparation seems to have a counterpart amongst those of the other preparation, indicating that peak A and peak B are entirely distinct enzymes. However, although less likely, another possibility for the different SDS-PAGE patterns would be that the peak A and B-associated polypeptides are related but might be subject to differential modification, such as phosphorylation, glycosylation, acetylation or specific proteolysis. Hence, in order to fully answer these questions, it will be necessary to obtain sequence information of the various polypeptides.

How do the enzyme preparations of our present study compare to plastid RNA polymerase activities that were previously reported? In maize, two activities could be partially separated by using a different procedure [68] and only one of them was studied further with regard to polypeptide structure. This latter activity was shown by protein sequencing to be related to the *E. coli* RNA polymerase [22, 23]. Two different RNA polymerase activities were likewise reported for pea chloroplasts [33], each with distinct transcription preference for the rrn versus *psbA* promoter. Since the two activities share immunochemically related polypeptides, it is possible that they represent two forms of one and the same enzyme that are associated with different sets of auxiliary polypeptides. Multiple plastid RNA polymerase activities were also reported for spinach, the major form of which seems to represent the *E. coli*-type enzyme based on its immunological relationship to the bacterial protein [34].

Interestingly, in none of these cases it was reported that highly purified plastid RNA polymerase is inhibited by the antibiotic rifampicin, although an inhibitory effect was previously shown for partially purified preparations [6]. A reasonable explanation for this discrepancy would be that the product of the plastid *rpoB* homologue contains sufficient amino acid substitutions within the region responsible for rifampicin binding in the bacterial β subunit [26]. The partial rifampicin sensitivity of crude enzyme preparations could be mediated by other proteins

present in such fractions. However, another possible explanation comes from our observation that there is a plastid-type-specific activity shift of peak A versus peak B (Fig. 1B). Since only peak B is sensitive against rifampicin but expressed very weakly in mature chloroplasts, inhibition is hardly detectable using more crude enzyme preparations from this plastid type. On the other hand, since peak B seems to represent the *E. coli*-like RNA polymerase form, one would expect rifampicin sensitivity in extracts from etioplasts or intermediate plastid types during greening. Indeed, the earlier experiments with maize which showed a partial inhibition by rifampicin were done with dark-grown seedlings that were then transferred to light in order to allow greening [6].

Recently, the possible existence of a T7-like plastid RNA polymerase consisting of a single 110 kDa polypeptide was reported for spinach chloroplasts [37]. Our present work using preparations from 4-day-old mustard seedlings did not substantiate these findings. However, similar to the situation in spinach, a second (minor) peak was noted upon heparin-Sepharose chromatography when 14-day-old mustard plants were used for chloroplast preparation, indicating the possible existence of an additional developmentally regulated RNA polymerase in mature tissue (unpublished data). It remains to be seen if the 110 kDa polypeptide reported for spinach is functional as a complete RNA polymerase *in vivo* or, alternatively, represents the catalytic core of a larger complex yet to be characterized. In any case, these findings stress the importance of analysing different plastid types of different developmental stage and/or tissue type in order to detect the full complement of proteins that together represent the organelle transcription apparatus [55, 31, 50]. In our present work we have investigated the peak A and peak B activities in different plastid types in seedlings of the same age (4 days old). It is conceivable that earlier in development at the proplastid stage, plastid transcription might be driven exclusively by the *E. coli*-like RNA polymerase B. Subsequent differentiation in the light then stimulates a shift towards plastid RNA polymerase A, which becomes dominant in the mature chloroplast. Although the exact sequence of events likely needs further refinement and modification, there are already data available that support this view. In barley, the *rpoBC* operon is expressed very early and the abundance of *rpoB* transcripts declines rapidly with further plastid development [1]. Likewise, transcript levels for the *rpo* genes from rice were found to decline with age, with only low levels of *rpoA*, $rpoC_1$ and $rpoC_2$ transcripts and virtually none for *rpoB* transcripts detectable in 10-day-old seedlings [27].

The maize plastid RNA polymerase which was identified as the *E. coli*-like enzyme by N-terminal sequencing was purified from plastids of plants which were grown 7–8 days in the dark after 16 h of illumination [22, 23, 68]. Hence, similar to the PT16 plastids of our present work, both peak A and peak B RNA polymerases might be present under these conditions in a roughly 1:1 ratio in maize as well. Indeed, two types of RNA polymerase activities were purified from these plastids of greening maize leaves [68], one of which (PF peak) was characterized further and identified as the *E. coli*-like enzyme. Although the nature of the other activity (BF peak) is still unknown, it might well represent a peak A-type RNA polymerase.

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References

1. Baumgartner BJ, Rapp JC, Mullet J: Plastid genes encoding the transcription/translation apparatus are differentially transcribed early in barley (*Hordeum vulgare*) chloroplast development. Evidence for selective stabilization of psbA mRNA. Plant Physiol 101: 781–791 (1993).
2. Bedbrook JR, Link G, Coen DM, Bogorad L, Rich A:

Maize plastid gene expressed during photoregulated development. Proc Natl Acad Sci USA 75: 3060–3064 (1978).
3. Bogorad L: Replication and transcription of plastid DNA. In: Bogorad L, Vasil IK (eds) The Molecular Biology of Plastids. Cell Culture and Somatic Cell Genetics, vol. 7A, pp. 93–117. Academic Press, San Diego, CA (1991).
4. Bogorad L, Vasil IK: The Molecular Biology of Plastids. Cell Culture and Somatic Cell Genetics, vol. 7A. Academic Press, San Diego, CA (1991).
5. Bogorad L, Vasil IK: The Photosynthetic Apparatus: Molecular Biology and Operation. Cell Culture and Somatic Cell Genetics, vol. 7B. Academic Press, San Diego, CA (1991).
6. Bogorad L, Woodcock CLF: Rifamycins: the inhibition of plastid RNA synthesis *in vivo* and variable effects on chlorophyll formation in maize leaves. In: Boardman NK, Linnane AW, Smillie RM (eds) Autonomy and Biogenesis of Mitochondria and Chloroplasts, pp. 92–97. North-Holland Publ. Co., Amsterdam (1970).
7. Bottomley W, Smith HJ, Bogorad L: RNA polymerases of maize: Partial purification and properties of the chloroplast enzyme. Proc Natl Acad Sci USA 68: 2412–2416 (1971).
8. Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 84: 248–254 (1976).
9. Bülow S, Link G: Sigma-like activity from mustard (*Sinapis alba* L.) chloroplasts conferring DNA-binding and transcription specificity to *E. coli* core RNA-polymerase. Plant Mol Biol 10: 349–357 (1988).
10. Bülow S, Reiss T, Link G: DNA-binding proteins of the transcriptionally active chromosome from mustard (*Sinapis alba* L.) chloroplasts. Curr Genet 12: 157–159 (1987).
11. Bünger W, Feierabend J: Capacity for RNA synthesis in 70 S ribosome-deficient plastids of heat-bleached rye leaves. Planta 149: 163–169 (1980).
12. Deng XW, Gruissem W: Control of plastid gene expression during development: the limited role of transcriptional regulation. Cell 49: 379–387 (1987).
13. DePamphilis CW, Palmer JD: Loss of photosynthetic and chlororespiratory genes from the plastid genome of a parasitic flowering plant. Nature 348: 337–339 (1990).
14. Dietrich D, Detschey S, Neuhaus H, Link G: Temporal and light control of plastid transcript levels for proteins involved in photosynthesis during mustard (*Sinapis alba* L.) seedling development. Planta 172: 393–399 (1987).
15. Eisermann A, Tiller K, Link G: *In vitro* transcription and DNA binding characteristics of chloroplast and etioplast extracts from mustarcd (*Sinapis alba*) indicate differential usage of the psbA promoter. EMBO J 9: 3981–3987 (1990).
16. Fong SE, Surzycki SJ: Chloroplast RNA polymerase genes of *Chlamydomonas reinhardtii* exhibit an unusual structure and arrangement. Curr Genet 21: 485–497 (1992).
17. Hallick RB, Lipper C, Richards OC, Rutter WJ: Isolation of a transcriptionally active chromosome from chloroplasts of *Euglena gracilis*. Biochemistry 15: 3039–3045 (1976).
18. Hartmann G, Honikel KO, Knusel F, Nuesch J: The specific inhibition of the DNA-directed RNA synthesis by rifamycin. Biochim Biophys Acta 145: 843–844 (1967).
19. Herrmann RG: Plant Gene Research. Springer-Verlag, Wien/New York (1992).
20. Herrmann RG, Westhoff P, Link G: Biogenesis of plastids in higher plants. In: Herrmann RG (ed), Cell Organelles, pp. 276–349. Springer-Verlag, Wien/Heidelberg/New York (1992).
21. Hess WR, Prombona A, Fieder B, Subramanian AR, Börner T: Chloroplast *rps15* and the *rpoB/C1/C2* gene cluster are strongly transcribed in ribosome-deficient plastids: evidence for a functioning non-chloroplast-encoded RNA polymerase. EMBO J 12: 563–571 (1993).
22. Hu J, Bogorad L: Maize chloroplast RNA polymerase: the 180-, 120- and 38-kilodalton polypeptides are encoded in chloroplast genes. Proc Natl Acad Sci USA 87: 1531–1535 (1990).
23. Hu J, Troxler RF, Bogorad L: Maize chloroplast RNA polymerase: The 78-kilodalton polypeptide is encoded by the plastid $rpoC_1$ gene. Nucl Acids Res 19: 3431–3434 (1991).
24. Hudson GS, Holton TA, Whitfeld PR, Bottomley W: Spinach chloroplast *rpoBC* genes encode three subunits of the chloroplast RNA polymerase. J Mol Biol 200: 639–654 (1988).
25. Hughes JE, Neuhaus H, Link G: Transcript levels of two adjacent chloroplast genes during mustard (*Sinapis alba* L.) seedling development are under differential temporal and light control. Plant Mol Biol 9: 355–363 (1987).
26. Igloi GL, Kössel H: The transcriptional apparatus of chloroplasts. Plant Sci 10: 525–558 (1992).
27. Kanno A, Hirai A: A transcription map of the chloroplast genome from rice (*Oryza sativa*). Curr Genet 23: 166–174 (1993).
28. Kidd GH, Bogorad L: A facile procedure for purification maize chloroplast RNA polymerase from whole cell homogenates. Biochim Biophys Acta 609: 14–30 (1980).
29. Kirk JTO: DNA-dependent RNA synthesis in chloroplast preparations. Biochem Biophys Res Commun 14: 393–397 (1964).
30. Klein RR, Mullet JE: Light-induced transcription of chloroplast genes. *psbA* transcription is differentially enhanced in illuminated barley. J Biol Chem 265: 1895–1902 (1990).
31. Krupinska K: Transcriptional control of plastid gene expression during development of primary foliage leaves of barley grown under a daily light-dark regime. Planta 186: 294–303 (1992).

32. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685 (1970).
33. Lakhani S, Khanna NC, Tewari KK: Two distinct transcriptional activities of pea (*Pisum sativum*) chloroplasts share immunochemically related functional polypeptides. Biochem J 286: 833–841 (1992).
34. Lerbs S, Bräutigam E, Mache R: DNA-dependent RNA polymerase of spinach chloroplasts: Characterization of sigma-like and alpha-like polypeptides. Mol Gen Genet 211: 459–464 (1988).
35. Lerbs S, Bräutigam E, Partier B: Polypeptides of DNA-dependent RNA-polymerase of spinach chloroplasts: Characterization by antibody-linked polymerase assay and determination of sites of synthesis. EMBO J 4: 1661–1666 (1985).
36. Lerbs S, Briat JF, Mache R: Chloroplast RNA polymerase from spinach: purification and DNA-binding proteins. Plant Mol Biol 2: 67–74 (1983).
37. Lerbs-Mache S: The 110-kDa polypeptide of spinach plastid DNA-dependent RNA polymerase: Single-subunit enzyme or catalytic core of multimeric enzyme complexes. Proc Natl Acad Sci USA 90: 5509–5513 (1993).
38. Little MC, Hallick RB: Chloroplast *rpoA*, *rpoB*, and *rpoC* genes specify at least three components of a chloroplast DNA-dependent RNA polymerase active in tRNA and mRNA transcription. J Biol Chem 263: 14302–14307 (1988).
39. Link G: Hybridization study of developmental plastid gene expression in mustard (*Sinapis alba* L.) with cloned probes for most plastid DNA regions. Plant Mol Biol 3: 243–248 (1984).
40. Link G: Plastid differentiation: organelle promoters and transcription factors. In: Nover L (ed) Plant Promoters and Transcription Factors, pp. 63–83. Springer-Verlag, Berlin/Heidelberg (1994).
41. Link G: Photoregulated development of chloroplasts. In: Bogorad L, Vasil IK (eds) The Photosynthetic Apparatus: Molecular Biology and Operation. Cell Culture and Somatic Cell Genetics, vol. 78, pp. 365–386. Academic Press, San Diego, CA (1991).
42. Merril CR, Goldman D, Van Keuren MR: Silver staining methods for polyacrylamide gel electrophoresis. In: Grossman L, Moldave K (eds) Method in Enzymology, pp. 230–239. Academic Press, New York (1983).
43. Morden CW, Wolfe KH, DePamphilis CW, Palmer JD: Plastid translation and transcription genes in a non-photosynthetic plant: Intact, missing and pseudo genes. EMBO J 10: 3281–3288 (1991).
44. Mullet JE: Chloroplast development and gene expression. Annu Rev Plant Physiol Plant Mol Biol 39: 475–502 (1988).
45. Mullet JE, Klein RR: Transcription and RNA stability are important determinants of higher plant chloroplast RNA levels. EMBO J 6: 1571–1579 (1987).
46. Neuhaus H, Link G: The chloroplast psbK operon from mustard (*Sinapis alba* L.): Multiple transcripts during seedling development and evidence for divergent overlapping transcription. Curr Genet 18: 377–383 (1990).
47. Ohyama K, Fukuzawa H, Kohchi T, Shirai H, Sano T, Sano S, Umesono K, Shiki S, Takeuchi M, Chang Z, Aota S, Inokuchi H, Ozeki H: Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. Nature 322: 572–574 (1986).
48. Purton S, Gray JC: The plastid *rpoA* gene encoding a protein homologous to the bacterial RNA polymerase alpha subunit is expressed in pea chloroplasts. Mol Gen Genet 217: 77–84 (1989).
49. Rajasekhar VK, Sun E, Meeker R, Wu B-W, Tewari KK: Highly purified pea chloroplast RNA polymerase transcribed both rRNA and mRNA genes. Eur J Biochem 195: 215–228 (1991).
50. Rapp JC, Baumgartner BJ, Mullet J: Quantitative analysis of transcription and RNA levels of 15 barley chloroplast genes. Transcription rates and mRNA levels vary over 300-fold; Predicted mRNA stabilities vary 30-fold. J Biol Chem 267: 21404–21411 (1992).
51. Reiss T, Link G: Characterization of transcriptionally active DNA-protein complexes from chloroplasts and etioplasts of mustard (*Sinapis alba* L.). Eur J Biochem 148: 207–212 (1985).
52. Rochaix JD: Post-transcriptional steps in the expression of chloroplast genes. Annu Rev Cell Biol 8: 1–28 (1992).
53. Rodermel SR, Bogorad L: Maize plastid photogenes: mapping and photoregulation of transcript levels during light-induced development. J Cell Biol 100: 463–476 (1985).
54. Ruf M, Kössel H: Structure and expression of the gene coding for the alpha-subunit of DNA-dependent RNA polymerase from the chloroplast genome of *Zea mays*. Nucl Acids Res 16: 5741–5753 (1988).
55. Schrubar H, Wanner G, Westhoff P: Transcriptional control of plastid gene expression in greening sorghum seedlings. Planta 183: 101–111 (1991).
56. Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayashida N, Matsubayashi T, Zaita N, Chungwongse J, Obokata J, Yamaguchi-Shinozaki K, Deno H, Kamogashira T, Yamada K, Kusuda J, Takaiwa F, Kato A, Tohdoh N, Shimada H, Sugiura M: The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. EMBO J 5: 2043–2049 (1986).
57. Sijben-Müller G, Hallick RB, Alt J, Westhoff P, Herrmann RG: Spinach plastid genes for initiation factor IF-1, ribosomal S11 and RNA polymerase alpha-subunit. Nucl Acids Res 14: 1029–1044 (1986).
58. Smith HJ, Bogorad L: The polypeptide subunit structure of the DNA-dependent RNA polymerase of *Zea mays* chloroplasts. Proc Natl Acad Sci USA 71: 4839–4842 (1974).

59. Sugiura M: The chloroplast genome. Plant Mol Biol 19: 149–168 (1992).
60. Surzycki SJ: Genetic function of the chloroplast of *Chlamydomonas reinhardtii*: effect of rifampin on chloroplast DNA-dependent RNA polymerase. Proc Natl Acad Sci USA 63: 1327–1334 (1969).
61. Tewari KK, Goel A: Solubilization and partial purification of RNA polymerase from pea chloroplasts. Biochemistry 22: 2142–2148 (1983).
62. Thompson WF, Everett M, Polans NO, Jorgensen RA, Palmer JD: Phytochrome control of RNA levels in developing pea and mung-bean leaves. Planta 158: 487–500 (1983).
63. Tiller K, Link G: Sigma-like transcription factors from mustard (*Sinapis alba* L.) etioplast are similar in size to, but functionally distinct from, their chloroplast counterparts. Plant Mol Biol 21: 503–513 (1993).
64. Tiller K, Link G: Phosphorylation and dephosphorylation affect functional characteristics of chloroplast and etioplast transcription systems from mustard (*Sinapis alba* L.). EMBO J 12: 1745–1753 (1993).
65. Tiller K, Eisermann A, Link G: The chloroplast transcription apparatus from mustard (*Sinapis alba* L.). Evidence for three different transcription factors which resemble bacterial sigma factors. Eur J Biochem 198: 93–99 (1991).
66. Woodbury NW, Dobres M, Thompson WF: The identification and localization of 33 pea chloroplast transcription initiation sites. Curr Genet 16: 433–445 (1989).
67. Yepiz-Plascencia GM, Radebaugh CA, Hallick RB: The *Euglena gracilis* chloroplast *rpoB* gene. Novel gene organization and transcription of the RNA polymerase subunit operon. Nucl Acids Res 18: 1869–1878 (1990).
68. Zaitlin D, Hu J, Bogorad L: Binding and transcription of relaxed DNA templates by fractions of maize chloroplast extracts. Proc Natl Acad Sci USA 86: 876–880 (1989).