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Hybridization study of developmental plastid gene expression in mustard (*Sinapsis alba* L.) with cloned probes for most plastid DNA regions

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Summary

An approach to assess the extent of developmental gene expression of various regions of plastid (pt)DNA in mustard (*Sinapis alba* L.) is described. It involves cloning of most ptDNA regions. The cloned regions then serve as hybridization probes to detect and assess the abundance of complementary RNA sequences represented in total plastid RNA. By comparison of the hybridization pattern observed with plastid RNA from either dark-grown or light-grown plants it was found that many ptDNA regions are constitutively expressed, while several 'inducible' regions account for much higher transcript levels in the chloroplast than in the etioplast stage. The reverse situation, i.e. 'repressed' regions which would account for higher transcript levels in the etioplast, was not observed. The hybridization results obtained with RNA from 'intermediate-type' plastids suggest that transient gene expression is a common feature during light-induced chloroplast development. The time-course of gene expression differs for various ptDNA regions.

Introduction

Plastid development in higher plants is a complex process that involves the cooperative expression of both nuclear and plastid genes (for reviews: 3, 9). The normal developmental program of plastid gene expression depends on the external factor light, which operates to a large extent through the phytochrome photoreceptor system (8). While the initial mechanisms of light action on plant gene expression are still unresolved, it is well-established that specific mRNAs for plastid proteins accumulate in response to light. Among those light-induced mRNAs which have been studied in detail by using recombinant DNA techniques are the nuclear-encoded transcripts for the small subunit (SS) of ribulose-1.5-bisphosphate carboxylase (RuBPCase) and for the light-harvesting chlorophyll a/b binding protein (4, 11, 13). By using similar techniques, it was shown that also a major chloroplast-encoded transcript, i.e. the mRNA for the M_r 32 000 photosystem II (PSII) protein, is under light control (1, 6, 7, 10). This plastid mRNA is abundant in light-grown plants but is present in low levels, if at all, in dark-grown plants. The accumulation of a second major transcript of a plastid gene, the mRNA for the large subunit (LS) of RuBPCase, was likewise found to be dependent on light in pea seedlings (11). In mustard cotyledons, however, high levels of this mRNA were also detected in dark-grown tissue (7). With respect to the possible mechanisms involved in light-dependent plastid development in mustard, it seemed important to investigate whether any other light-induced transcripts encoded by ptDNA genes can be detected. In the present study this problem is approached by DNA/RNA hybridization, using cloned fragments from almost the entire mustard ptDNA circle as probes.

Abbreviations

kb	kilobases
ptDNA	plastid DNA

Material and methods

Isolation of mustard ptDNA and physical mapping procedures, using restriction endonucleases SalI, XhoI, PstI, SmaI and BglI (Bethesda Research Laboratories), were as described (5). Cloning of ptDNA fragments in pBR322 was by procedures similar to those described previously (6). Clones were identified by cross-hybridization with purified mustard ptDNA fragments and by size comparison of subfragments. Detailed restriction maps of cloned fragments were obtained by single and double digestions with various restriction enzymes and cross-hybridization of overlapping fragments (5).

RNA preparation. Mustard seedlings were grown on vermiculite for 5 days either in darkness or, alternatively, under continuous white light before cotyledons were harvested, and either etioplasts or chloroplasts were prepared as described (2, 7). Other mustard seedlings were grown for 4 days in darkness and then transferred to light for 18 hr before harvesting of cotyledons and preparation of these 'intermediate-type' plastids. Plastid RNA was prepared and labelled *in vitro* with ^{32}P by polynucleotide kinase as described (1, 7).

Gel electrophoresis of cloned ptDNA fragments, blotting to nitrocellulose (12), and hybridization conditions using equal amounts of radioactive total ptRNA in each experiment, were as described (7).

Results and discussion

The following approach was used for investigating developmental changes in the pattern of plastid RNA species:

(1) Mustard chloroplast DNA was mapped and segments from nearly all regions of the DNA circle were cloned in bacterial plasmids.

(2) The cloned mustard ptDNA fragments were digested with restriction enzymes. The resulting subfragments of known map position were separated by electrophoresis, blotted onto nitrocellulose sheets, and were then used as hybridization probes for the detection of specific transcripts from these regions. Each 'developmental blot' contained standardized sets of DNA fragments from 12 selected plasmids, which together comprised almost the entire ptDNA circle.

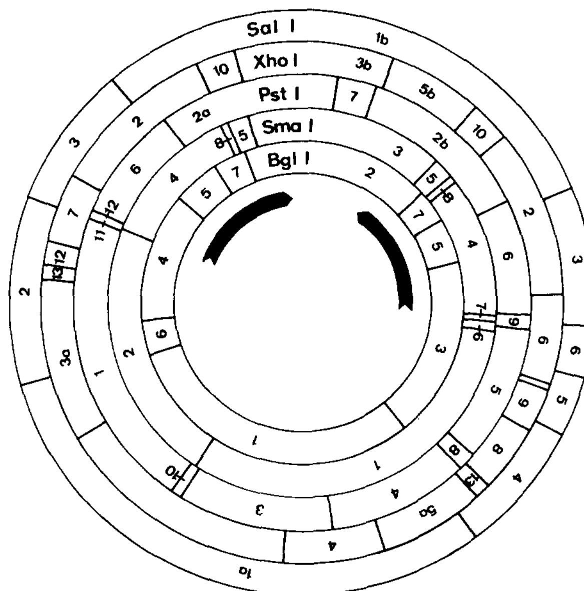

Fig. 1. Restriction endonuclease map of mustard plastid (pt)DNA. Recognition sites for SalI, XhoI, PstI, SmaI, and BglI are presented. Arrows: The two copies of the inverted repeat. The total size of the ptDNA circle is 157 kb (5).

(3) Plastid RNA obtained from either dark-grown or light-grown seedlings was prepared, labelled with ^{32}P, and hybridized to the ptDNA fragments immobilized to nitrocellulose.

Figure 1 shows a detailed restriction map of mustard ptDNA, giving the recognition sites for SalI, XhoI, PstI, SmaI, and BglI. Most of the Pst fragments (except Pst2a and Pst3) were cloned in pBR322, and cloned fragments were further mapped, using additional restriction enzymes (Fig. 3). In addition, a clone that contains an EcoRI generated fragment Eco2, spanning the region between Pst1 and Pst6, was included to supplement the set of Pst clones. Table 1 shows the cloned ptDNA segments as well as their subfragments used for the present analysis.

Figure 2 illustrates the pattern of DNA bands upon electrophoretic separation of the cloned (sub)-fragments. Upon blotting to nitrocellulose, the DNA fragments of three different gels run under standardized conditions served as hybridization probes for RNA obtained from three different plastid types: (A) etioplasts obtained from dark-grown plants; (B) 'intermediate-type' plastids from dark-grown plants transferred to light for 18 h; (C) chlo-

Table 1. Plasmids and ptDNA fragments used for DNA/RNA hybridization analysis.

	1	2	3	4	5	6	7	8	9	10	11	12
Plasmid pSA	303	530	340	340	364	317	318	308	452	304	335	211
Insert/ Size (kb)	Pst2a 18.5	Pst1 30.0	Pst4 15.0	Pst4 15.0	Pst5 12.8	Pst6 12.5	Pst7 5.0	Pst8 2.75	Pst9 2.15	Pst10 1.6	Pst11 1.2	Eco2 6.4
Restriction enzymes used	PstI/BamHI	PstI/EcoRI	PstI/BamHI/HindIII	PstI/BglII	PstI/BamHI/XhoI	PstI/BglI/HindIII/XhoI	PstI	PstI	PstI	PstI	PstI	EcoRI/PstI
Insert fragments/ Size (kb)												
a	7.2	4.8	3.3	7.5	4.3	5.6	5.0	2.75	2.15	1.6	1.2	3.4
b	3.5	4.2	2.9	3.5	2.8	3.55						1.2
c	3.0	2.7	2.6	2.0	1.9	1.9						0.9
d	1.8	2.5	2.2	1.6	1.5	1.45						0.8
e	1.6	2.1	2.05	0.4	1.3							
f	1.35	1.85	1.5	0.3	0.6							
g		1.7			0.45							
h		1.5										
i		1.15										
j		0.85										
k		0.8										
l		0.6										

roplast from plants grown under continuous light.

The pattern of autoradiographic bands obtained in all three experiments reflects specific DNA/RNA hybridization since there are signals only at the position of certain ptDNA bands and there are no signals due to vector DNA fragments. The intensity of hybridization signals reflects the relative frequency of transcripts. In Fig. 2C (chloroplast RNA), the most intensive signals are obtained for DNA fragments which hybridize to rRNAs (Track 1, bands a and c), to RuBPCase(LS)-mRNA (Track 2, band b), and to the mRNA for the M_r 32 000 PSII protein (Track 5, band d; track 9, band a). These RNA species were previously shown to be the most abundant chloroplast RNAs (7).

With chloroplast RNA (Fig. 2C), additional strong hybridization signals are detected, which correspond to positions on ptDNA fragments Pst4 (Track 3, band d; track 4, band b) and Eco2 (Track 12, band a). Less intense hybridization signals are found with fragments from most ptDNA regions analyzed, except for Pst8 and Pst10, which do not generate detectable signals. The hybridization data obtained with chloroplast RNA are schematically presented in Fig. 3 along with restriction maps of several of the ptDNA fragments used.

The hybridization pattern with etioplast RNA (Fig. 2A) shows strong signals at the position of the rRNA genes and the RuBPCase(LS) gene. But unlike with chloroplast RNA, the signals obtained for the gene for the M_r 32 000 PSII protein (Track 5, band d; track 9, band a) are very weak. This confirms the previous findings on the light regulation of this gene (7). Most other signals are of similar relative intensity as in the experiment with chloroplast RNA (Fig. 2C), except for the bands corresponding to Pst11 (Track 11) and Eco2 (Track 12), which are present only weakly (Track 12, band a) or not detectable. There is no relative increase in the intensity of any of the hybridization bands obtained with etioplast RNA as compared to chloroplast RNA.

The hybridization of cloned ptDNA fragments with RNA from 'intermediate-type' plastids of plants kept in the light for 18 hr (Fig. 2B) again gives rise to strong signals at the positions of rRNA genes and the RuBPCase(LS) gene. The signals of the gene for the M_r 32 000 PSII protein (Track 5, band d; track 9, band a) are more intensive than with etioplast RNA (Fig. 2A) but less intensive than with chloroplast RNA (Fig. 2C). Other signals obtained in this experiment can be classified as fol-

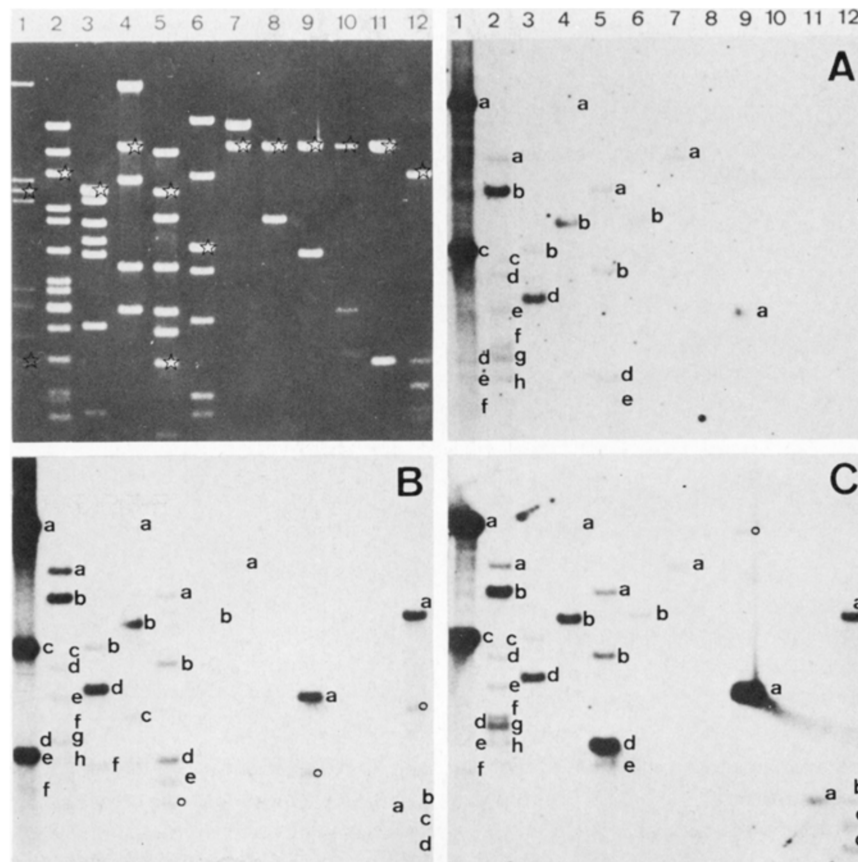

Fig. 2. Analysis of expressed mustard ptDNA regions by hybridization of cloned ptDNA fragments to plastid RNA. The photograph of the stained gel (upper left square) shows the separated fragments of cloned ptDNA regions. Tracks are numbered 1–12, which refers to the plasmids and their ptDNA inserts summarized in Table 1. The table also gives the combinations of restriction enzymes used for generating the (sub)fragments as well as the designation (small letters) and size (kb) of each ptDNA fragment. The DNA bands marked with asterisks on the gel photograph represent pBR322 fragments (in tracks 3 and 12, the largest ptDNA fragment comigrates with a pBR322 fragment). In each track one μg of DNA was loaded, except for track 1, which contained 0.1 μg DNA to reduce the intensity of the strong rRNA signals.

A. Hybridization signals of etioplast RNA with the blotted ptDNA fragments shown in tracks 1–12.

B. Hybridization signals of RNA from 'intermediate-type' plastids of plants illuminated for 18 h.

C. Hybridization signals of chloroplast RNA.

The small letters refer to hybridization bands with ptDNA fragments of Table 1. The map position of these fragments is given in Fig. 1 and Fig. 3. The small circles mark several hybridization signals of unknown origin, which were not included in the analysis.

lows: (1) Bands which are of similar relative intensity as with chloroplast RNA (e.g. track 12, band a); (2) Bands which are of low intensity or absent as with etioplast RNA (e.g. track 11, band a; track 12, bands b, c, d); (3) Bands which are more prominent than with RNA from any of the two other plastid types (Track 1, band e within fragment Pst2a; track 2, band a within Pst1; track 3, band f and track 4, band c within Pst4). The remaining bands are of similar relative intensity as with RNA from both other two plastid types.

Figure 3 summarizes the results of the hybridization experiments shown in Fig. 2 and gives details on the map location of the ptDNA regions which hybridize to plastid RNAs. This schematic representation shows that transcribed genes are located within all three major regions of the mustard ptDNA molecule, i.e. within the large and small

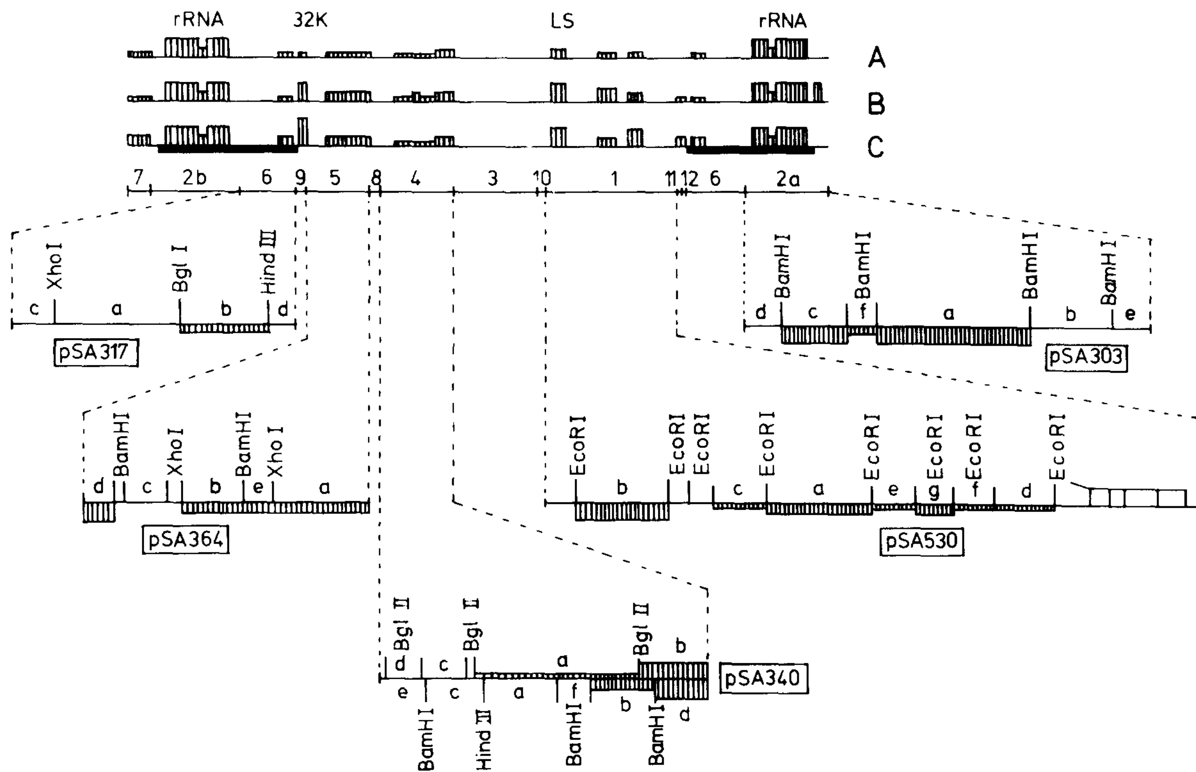

Fig. 3. Mustard ptDNA regions expressed during plastid development. The mustard ptDNA molecule linearized in the small single-copy region is shown as the central horizontal line. Numbers refer to the Pst fragments. Several of these fragments are drawn below on a larger scale, and recognition sites for restriction enzymes are presented. The small letters refer to mapped subfragments of Table 1 and Fig. 2. The relative intensity of hybridization signals obtained with chloroplast RNA (Fig. 2C) is represented by the height of the hatched fields for each of these subfragments.

A summary of the hybridization results of Fig. 2 is given in the upper portion of Fig. 3. On three horizontal lines (A–C) are indicated the positions of ptDNA regions which give rise to hybridization signals with plastid RNA. A. Signals with etioplasts RNA; B. Signals with RNA from 'intermediate-type' plastids of plants illuminated for 18 h; C. Signals with chloroplast RNA. The relative intensity of signals is given by the height of the hatched boxes.

rRNA: Coding region for rRNAs; 32K: Coding region for the M_r 32 000 PSII protein; LS: Coding region for RuBPCase(LS). The heavy horizontal bars below line C represent the two copies of the inverted repeat.

single-copy regions as well as in the two copies of the inverted repeat.

One principal limitation of the hybridization approach that has been used here is the lack of information on the precise map location of most plastid genes. For instance, several clustered genes on a single DNA fragment would account for a hybridization signal of an intensity that represents the sum of hybridization intensities of the individual genes. It is conceivable that increased hybridization to one of these genes could be compensated by decreased hybridization to an adjacent gene. Therefore, it is likely that the observed differences in hybridization with RNA from the various plastid forms do not fully reflect all stage-specific differences of these ptRNA populations. In particular, very rare RNA species may have escaped detection. This stresses the importance of detailed gene mapping studies, e.g. by R-loop analysis or S1 mapping. By using these techniques, we have recently been able to define the precise positions of a number of mustard plastid genes (G. Dietrich, T. Hönig, H. Falk & G. Link, to be published). The availability of probes specific for single genes is a prerequisite for extending the present developmental study at the level of single RNA species, as has already been demonstrated for the mRNAs for RuBPCase(LS) and the M_r 32 000 PSII protein (6, 7, 14). Despite these

inherent difficulties, the present hybridization study is a suitable approach towards defining the regions on the mustard ptDNA molecule which account for (developmentally regulated) plastid transcripts.

Since the intensity of DNA/RNA hybridization bands in Fig. 2 reflects the abundance of the hybridized RNA species, the developmental status of various plastid forms can be analyzed at the level of plastid RNA. As shown in Figs. 2 and 3, most, if not all, hybridization signals with chloroplast RNA are also detected with etioplast RNA. However, at least several of the hybridizing RNA species are represented in much lower relative amounts in total etioplast RNA than in chloroplast RNA. This indicates that most plastid genes are constitutively expressed during plastid development, while the expression of a subset of plastid genes is induced during the transition from the etioplast to the chloroplast stage. The reverse situation, i.e. 'repressed' genes which give rise to higher RNA levels in etioplasts than in chloroplasts, has not been observed in the present study.

Obviously, different (groups of) RNA species are accumulated with different time-course during light-dependent plastid development. The mRNA for the M_r 32 000 PSII protein gives rise to a hybridization signal of intermediate intensity with RNA from ('intermediate-type') plastids of plants illuminated for 18 h as compared to the signals obtained with either chloroplast or etioplast RNA (Fig. 2, track 5, band d, and track 9, band a). However, some hybridization signals with RNA of 'intermediate-type' plastids are already of similar (high) intensity as with chloroplast RNA (Track 12, band a), while other signals are still of similar (low) intensity as with etioplast RNA (Track 12, bands b–d). The hybridization pattern with RNA of 'intermediate-type' plastids (Fig. 2B) shows several signals of higher relative intensity than with either chloroplast or etioplast RNA. This indicates that transient gene expression does occur during light-induced plastid development. It will be the aim of future studies to investigate in more detail the time-course of RNA species which are transiently accumulated during plastid development. The analysis of genes coding for coordinately induced plastid RNA species can be expected to provide clues to the regulatory mechanism(s) involved in their expression.

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