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Expression of the Large ATP Synthase Gene Cluster in Spinach Plastids during Light-Induced Development[1]

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ABSTRACT

The large ATP synthase gene cluster in spinach (*Spinacia oleracea*) plastids encodes four of the six chloroplast-encoded ATP synthase subunits. Expression of this cluster was examined to determine its response to light-induced plastid development. Spinach plastid transcripts were isolated from etiolated tissues, etiolated tissues exposed to 24 h of light, young (1–3 cm) leaves, and mature (8–10 cm) leaves. Transcript levels were examined from each developmental stage as a function of either the quantity of total RNA or gene dosage. The relative transcriptional activity of this gene cluster at each of the four developmental stages was also investigated. The stability of these transcripts was deduced by comparing the transcriptional activity with steady-state transcript levels. During the initial 24 h of light-induced development of an etioplast to a chloroplast, transcription decreases in conjunction with increased transcript stability. Transcriptional activity of this cluster per genome then increases between the 24-h and young stages, with a concomitant decrease in the stability of the transcripts. As the young chloroplast matures, the transcripts from this cluster again become markedly more stable, and the transcription of this set of genes declines. Therefore, the regulation of the expression of this cluster is dependent upon a complex interaction between transcriptional and posttranscriptional factors throughout light-induced plastid development.

During the first 24 h of exposure to light, etioplasts in photosynthetic tissues develop into chloroplasts. This developmental process is marked by the organization of thylakoid membranes in conjunction with the onset of photosynthesis (1, 3, 5, 13, 16, 17, 21). Control of the expression of thylakoid membrane protein-encoding genes is crucial to chloroplast biogenesis during the course of this light-induced development (8, 17).

The degree to which light-induced development affects plastid transcription and transcript levels varies depending upon the plant species and the genes being studied. Light-modulated effects on specific transcript abundance have been observed in barley (6, 14, 15, 18), mustard (16), maize (20), and numerous other plant species (reviews in refs. 8 and 17). The process of light-induced development is complex, and its molecular basis is not fully understood.

A thorough study of the molecular effects of light-induced plastid development is essential to understanding the response of plastid genes to developmental signals. We have chosen to study light-induced effects on the expression of a multigenic locus encoding the large ATP synthase gene cluster found in spinach (*Spinacia oleracea*) plastids. ATP synthase is a thylakoid-bound complex that becomes active during development (2, 7).

Four of the six plastid-encoded subunits of the ATP synthase complex are located in the large ATP synthase gene cluster on the plastid genome (11, 12, 22). This cluster encodes, in order of transcription, the CF_0-IV (atpI), CF_0-III (atpH), CF_0-I (atpF), and CF_1-α (atpA) subunits. A diagram of this gene cluster is shown in Figure 1. Cotranscription of these genes is followed by splicing (atpF contains a group II intron) and numerous endo- and exonucleolytic cleavages. The order and mechanisms of these posttranscriptional processing events are currently being investigated. The end result of these complex processing steps is approximately 30 individual transcripts (11, 12; N. Stollar and M.J. Hollingsworth, unpublished data). Two other ATP synthase genes are also encoded on the plastid genome. These are CF_1-β and CF_1-ϵ, which make up the small ATP synthase gene cluster (10). The remaining three ATP synthase subunits, CF_1-γ, CF_1-δ, and CF_0-II, are nuclear encoded.

It has been shown that, for many of the genes in spinach plastids, the relative transcriptional activity does not change significantly during light-induced development (4). However, the absolute level of many of the transcripts does change, often by as much as 20-fold. These observations imply that the steady-state levels of many spinach chloroplast transcripts are under posttranscriptional control (3).

Light-induced developmental effects on the expression of the large ATP synthase gene cluster in spinach plastids have not been previously studied. We investigated the expression of these loci by analysis of transcripts from spinach plastids at four different stages of development: etiolated, 24-h illuminated, young, and mature plants. Here, we present evidence that transcriptional activity and RNA stability are both significant factors in light-induced developmental regulation of the expression of this gene cluster. The relative influence of each factor, however, varies with the particular stage of development.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea*) seeds were germinated in vermiculite and then transferred and grown hydroponically to obtain young and mature leaves. Young leaves were collected

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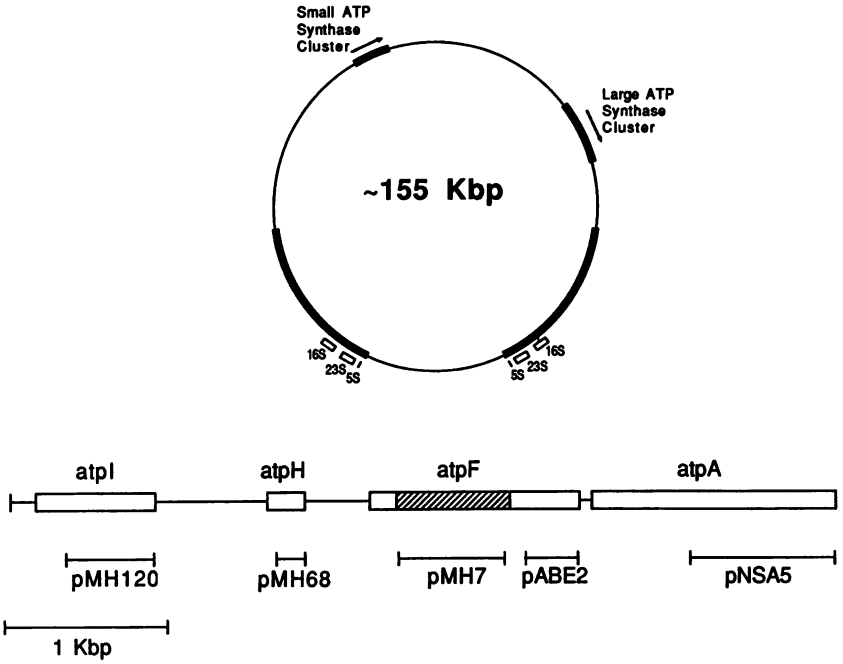

Figure 1. Organization of the spinach plastid large ATP synthase gene cluster. The entire spinach plastid genome is outlined as a circle at the top of this figure. The locations of the large and small ATP synthase gene clusters relative to the inverted repeat regions (heavy lines) are noted. A diagram of the large ATP synthase gene cluster is drawn below the genome. Transcription is from left to right. Genes are shown as open boxes. The atpF intron is represented by a shadowed box. Regions from the cluster that were cloned and used to generate probes are diagrammed as lines below the cluster; clone names are noted beneath the probe lines. Bar at the bottom left represents 1 kb.

when 1 to 3 cm in length, and mature leaves were collected when 8 to 10 cm in length. Etiolated seedlings were produced by germination and growth in vermiculite in complete darkness for 7 d at room temperature. The etiolated tissues were collected under dim, green filtered light. Twenty-four-hour illuminated plants were first germinated and grown in vermiculite in the dark for 6 d and then transferred to constant illumination for 24 h, both at room temperature. All plant tissues were collected on ice into cold GR[2] buffer (330 mM sorbitol, 50 mM Hepes [KOH] pH 8, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM sodium ascorbate). Etioplasts were collected in GR buffer with the sorbitol increased to 450 mM.

Because of the growth pattern of *S. oleracea*, the majority of the plastids isolated from etiolated and 24-h plants are derived from cotyledonous tissues. Cotyledons in spinach continue to develop and become fully photosynthetically competent as the plant matures. The earliest stages of plastid development in cotyledons parallel those in leaf tissue (13). In this study, we utilized plastids from cotyledons in the earliest stages of greening as models for the development that is concurrently taking place in leaf tissues.

Plastid Isolation

Plastids were isolated on Percoll step gradients as described by Orozco et al. (19). The concentration of sorbitol was increased to 450 mM in the Percoll during etioplast isolation. Intact plastids were divided into aliquots, immediately frozen, and stored at −75°C. Plastids isolated for use in run-on transcription assays were kept on ice in GR buffer. A portion of each plastid preparation was put aside and counted manually with a hemocytometer to determine plastid concentration.

[2] Abbreviations: GR, grinding; SSC, standard sodium citrate.

Nucleic Acid Isolation

Nucleic acids were isolated from frozen aliquots of plastids by lysis in the presence of 50 mM sodium acetate (pH 5.6), 1% (w/v) SDS. During RNA isolation, 5 mM aurintricarboxylic acid (9) or 4 M guanidinium thiocyanate was also included. Phenol/chloroform extraction was performed simultaneously with thawing and lysis of the samples. An additional phenol/chloroform extraction, followed by ethanol precipitation, resulted in pelleted nucleic acids, which were then resuspended in diethyl pyrocarbonate-treated H_2O. RNA concentrations were determined by spectrophotometry and/or gel electrophoresis.

Determination of Relative Plastid DNA Levels

Nucleic acids were isolated as described above. Etioplast nucleic acids were resuspended with H_2O to 5 μL/1 × 10^8 plastids; all others were resuspended to 5 μL/1 × 10^7. DNAs were prepared for blotting as follows. Nucleic acids from each stage of development (5 μL; etioplast concentration was a factor of 10 higher than the others for ease of detection) were diluted to 100 μL with H_2O. NaOH was added to a final concentration of 0.4 M NaOH. Samples were boiled for 10 min and quickly chilled in ice H_2O. The volume was brought up to 1.5 mL using a cold solution of 40 mM Tris (acetic acid), 1 mM EDTA, 20 mM sodium acetate (pH 7.4). A dot blot apparatus was fitted with a Zetaprobe (Bio-Rad) membrane, the wells were washed with 0.4 M NaOH, and denatured DNAs were applied. After DNAs were applied, the wells were again rinsed with 0.4 M NaOH. The Zetaprobe was air dried, and DNAs were permanently fixed to the membrane by baking at 80°C for 2 h. Membranes were prehybridized using a solution of 50% (v/v) formamide, 6× SSC (20× SSC: 3 M NaCl, 0.3 M sodium citrate), 1% (w/v) SDS, 0.1% (w/v) each of Ficoll, PVP, BSA, and degraded

herring sperm DNA (Sigma). Prehybridization was performed for a minimum of 4 h at 65°C. Hybridization was performed in the same solution with the addition of a uniformly ^{32}P-radiolabeled RNA probe transcribed from the atpI clone (Fig. 1). The probe was allowed to hybridize for 8 h at 42°C. Blots were then washed twice, each for 20 min at 42°C, using 2× SSC and 0.2% (w/v) SDS. During the second wash, the temperature was increased to 65°C. Two additional washes at 65°C followed, again for 20 min each, using 0.1× SSC and 0.01% (w/v) SDS. Liquid scintillation counting of excised dots and/or densitometry of the autoradiogram was performed to determine relative amounts of hybridization.

Northern Analysis

Nucleic acids were isolated in the presence of aurintricarboxylic acid or guanidinium thiocyanate as described above. RNA was subjected to electrophoresis in a 1% (w/v) agarose, 5.4% (v/v) formaldehyde gel. Ethidium bromide staining of one plastid RNA lane was performed to verify RNA integrity and to visualize size standards. RNAs were then blotted onto Nitroplus 2000 (MSI) using 10× SSC. Transferred RNAs were permanently fixed to the membrane by baking at 80°C for 2 h under a vacuum. The prehybridization solution consisted of 50% (v/v) formamide, 5× SSC, 0.1% (w/v) SDS, 0.02% (w/v) each of Ficoll, PVP, BSA, and degraded herring sperm DNA. The prehybridization was performed for a minimum of 4 h at 65°C. Hybridization was performed in the same solution with the addition of specific probes for 8 to 24 h at 42°C. The blots were washed as described for the dot blot analysis.

Hybridization to transcripts from the large ATP synthase gene cluster was in the presence of a mixture of the five probes diagrammed in Figure 1. These probes were complementary to each of the four open reading frames plus the single group II intron from this cluster. All five probes were included in the hybridization mixture to ensure that qualitative changes in the levels of any of the individual RNAs derived from this cluster could be detected. Northern analysis of RNAs from the small ATP synthase gene cluster was performed with a single probe complementary to both atpB and atpE. Further details concerning these probes are given in "Clones and Probes," below.

Total hybridization of RNAs at each of the four stages was quantitated by excision and liquid scintillation counting of individual lanes from the filter. To compare relative hybridization of each stage across several different experiments, each set of hybridization values was normalized to the stage within that set that had the least hybridization.

Clones and Probes

The sequence of the large ATP synthase gene cluster was previously reported (10, 12). The numbering system used here is that of Hudson et al. (12). This cluster, encoding the genes *atpI-atpH-atpF-atpA* (Fig. 1), was cloned into the multicloning site of SK+ Bluescript vector (Stratagene). Subclones of this initial clone (pJB6) were obtained for each gene of the cluster as follows. Plasmid MH68 has a 318-bp fragment from atpH that includes bases 2872 to 3100. The atpF clone contains the exon 2 sequence extending from bases 4450 to 4730 and is designated pABE2. The insert in pMH7 contains the atpF intron sequence 3700 to 4243. Plasmid NSA5 contains bases 5747 to 6414 of the atpA gene. Bases 1610 to 2154 of the atpI gene make up the insert of pMH17. The small ATP synthase gene cluster probe was generated from a clone that contained a 2.3-kbp region of the atpB/E gene (a generous gift of Drs. J. Narita and W. Gruissem). RNA probes were generated in vitro using the appropriate RNA polymerase promoter. The probe used to detect the 23S rRNA (data not shown) was a *Bam*HI/*Xho*I subclone containing 1460 bp of the 23S gene (generously given to us by Dr. J.-F. Briat).

Transcriptional Analysis

Run-on transcription was performed essentially as described by Klein and Mullet (15). Transcription was initiated by the addition of 5×10^6 plastids to a prewarmed tube containing the transcription mixture and 200 μCi of [α-^{32}P]-UTP (3000 Ci/mm, ICN). Plastids were lysed by pipeting up and down in the mixture several times. Transcription was allowed to proceed at 23°C for 10 min. The reaction was terminated by phenol/chloroform extraction.

Plasmid-derived DNAs corresponding to a single fragment encoding the entire large ATP synthase gene cluster were bound to nylon membranes (Zetaprobe). Run-on transcripts from an equivalent number of plastids from each developmental stage were hybridized to the DNAs. Hybridization and washing conditions were as given for the dot blot method. The quantity of DNA on the membranes was set such that the DNA was well in excess of any large ATP synthase gene cluster transcripts present in the hybridization mixture. Radioactive portions of the filters corresponding to the gene cluster were excised, and the radioactivity was determined by scintillation counting. Alternatively, relative hybridization of each lane was determined by densitometry of the autoradiogram. Previous experiments by N. Stollar (personal communication) showed that no RNAs are transcribed from the strand opposite the large ATP synthase gene cluster coding region.

RESULTS

Relative Gene Copy Numbers per Plastid

We wished to normalize each of our analyses of the expression of this cluster to the gene dosage at each developmental stage. Therefore, we first determined the relative gene copy number per plastid in each of four developmental stages. Hybridization to four different dot blots was averaged, and these values were utilized to determine the relative number of gene copies per plastid in each of the four stages. The results of these experiments are summarized in Figure 2A. It was determined that the relative number of gene copies per plastid increases by a factor of approximately 3 as the etioplast begins to green. The number of genomes per plastid then increases by another factor of approximately 2 as the chloroplast develops to the young stage. Genomes per plastid decrease only slightly, within experimental error, during the young to mature chloroplast transition.

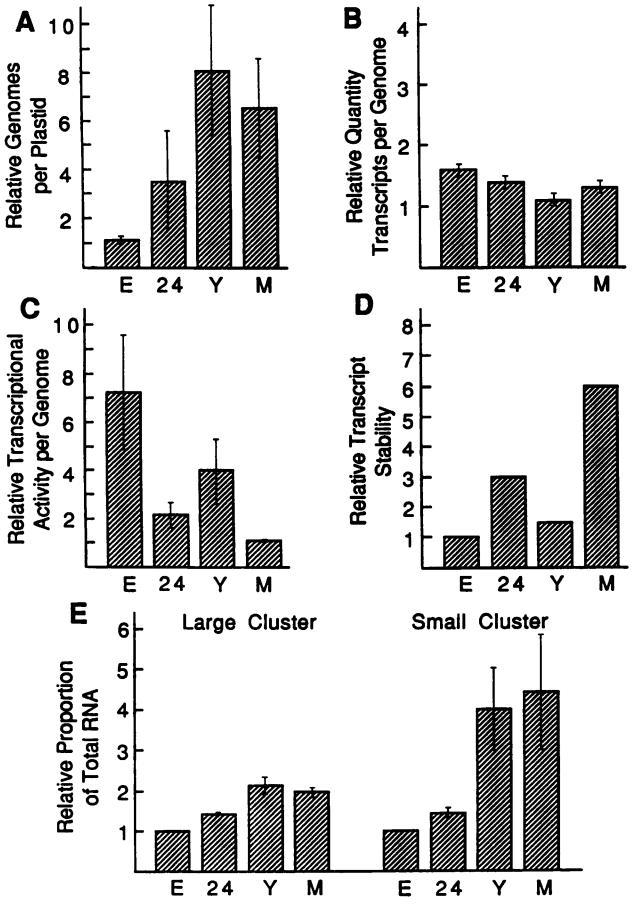

Figure 2. Factors that influence expression of the large ATP synthase gene cluster during development. Note that each of these histograms expresses the relative quantity of each of these factors at any given developmental stage compared with the other three stages. Vertical bars denote SE. A, Genomes per plastid (n = 4). B, Relative quantity of transcripts derived from the large ATP synthase gene cluster, normalized to gene dosage (n = 10). C, Relative transcriptional activity of the large ATP synthase gene cluster, normalized to gene dosage (n = 5). D, Relative stability of transcripts derived from the large ATP synthase gene cluster (derived from the data in B and C). E, Relative proportion of total plastid transcripts derived from the large (left, n = 6) and small (right, n = 5) ATP synthase gene clusters. E, Etioplast; 24, 24-h illuminated chloroplast; Y, young leaf chloroplast; M, mature leaf chloroplast.

Analysis of Transcripts Normalized to Gene Copy Number

Normalizing the amount of RNA per lane in northern analyses to plastid gene copy number enables visualization of the relationship between gene copy number and ATP synthase transcript levels at the various developmental stages. Northern analysis was performed using RNAs from each developmental stage. The quantities of RNAs loaded onto the gels were normalized to the young stage. For example, because etioplasts contain approximately 7-fold fewer genomes per plastid relative to young chloroplasts, RNAs from 7-fold more etioplasts than a given number of young chloroplasts were examined. The total hybridization to RNAs from each stage was quantitated by scintillation counting of each individually excised lane (see "Materials and Methods").

A typical autoradiogram from these experiments is shown in Figure 3. The degree of hybridization to individual transcripts varied somewhat between the four stages. However, quantitation of total hybridization at each stage revealed net differences between any two stages of no more than 50% (Fig. 2B). The variations in total hybridization, although small, are greater than the SE for these measurements.

Proportion of Total Plastid Transcripts Devoted to the Large ATP Synthase Gene Cluster

Equal quantities of plastid RNAs were examined by northern analysis to determine the relative proportion of transcripts from this gene cluster present during the four stages of light-induced development. Equivalent amounts of total plastid RNAs isolated from four developmental stages were hybridized to either a combination of five radioactive RNAs complementary to the large ATP synthase gene cluster (Figs. 1 and 4A) or a single probe complementary to the atpB/E cluster (Fig. 4B). Similar analyses were performed as controls with a probe from the 23S rRNA gene. The 23S RNA was found to be present in approximately equal percentages of the total plastid RNA populations in each of the four developmental stages (data not shown).

Results of the northern analyses show that the proportions of total plastid RNAs devoted to transcripts from the two plastid ATP synthase gene clusters may be coordinated during development (Figs. 2E and 4). The proportion of plastid transcripts devoted to both of the ATP synthase gene clusters remains relatively constant in etioplasts and 24-h chloroplasts

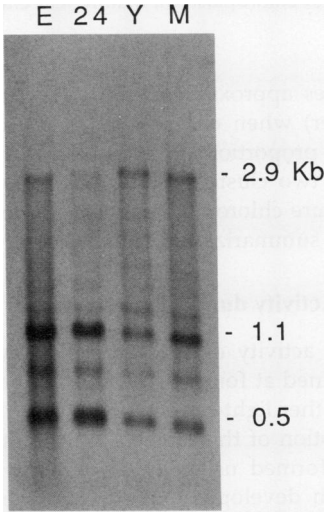

Figure 3. Northern analysis of transcripts derived from the large ATP synthase gene cluster during light-induced plastid development normalized to equal gene dosage. RNAs were extracted from plastids at each developmental stage. The quantity of RNA analyzed was normalized to the relative numbers of genomes per plastid (see text). Hybridization was performed with probes complementary to each of the genes and intron in the cluster. Numbers to the right designate transcript length. E, Etioplast; 24, 24-h illuminated chloroplast; Y, young leaf chloroplast; M, mature leaf chloroplast.

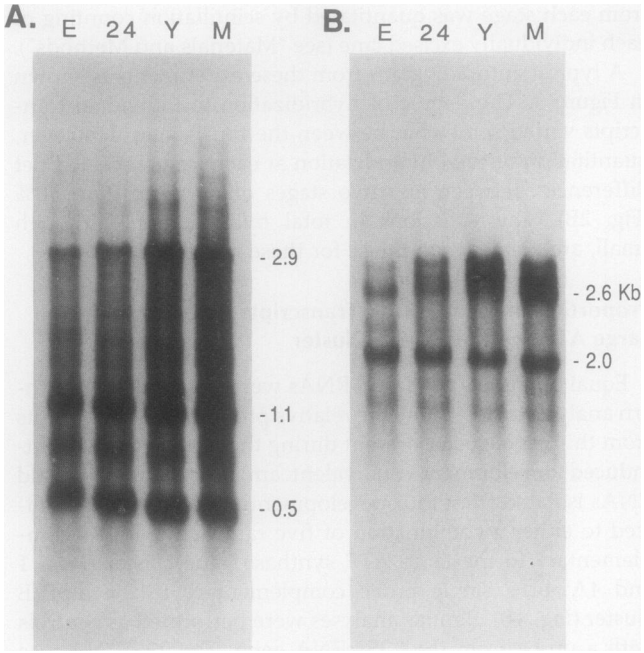

Figure 4. Northern analysis of transcripts derived from the large and small ATP synthase gene clusters during light-induced development on an equal RNA basis. RNAs extracted from plastids at four different developmental stages were separated by size and transferred to membrane filters. RNA (10 μg) from one stage are present in each lane. The filter was hybridized with probes corresponding to (A) five probes complementary to all four genes plus the intron of the large ATP synthase gene cluster (see Fig. 1) or (B) a single probe from the small ATP synthase gene cluster complementary to both the atpB and atpE genes. Numbers to the right designate transcript length. E, Etioplast; 24, 24-h illuminated chloroplast; Y, young leaf chloroplast; M, mature leaf chloroplast.

and then increases approximately 2- (large cluster) and 4-fold (small cluster) when comparing the 24-h with young chloroplasts. The proportions of total plastid transcripts derived from these two clusters remain relatively constant in the young to mature chloroplast transition. The quantitation of these results is summarized in Figure 2E.

Transcriptional Activity during Development

Transcriptional activity of the large ATP synthase gene cluster was examined at four different developmental stages to determine whether light-induced plastid development affects the transcription of this cluster. Transcriptional run-on analysis was performed using equal quantities of plastids derived from each developmental stage in the presence of [α-^{32}P]UTP (see "Materials and Methods"). The resulting radiolabeled transcripts were hybridized to excess quantities of a single DNA fragment encoding the large ATP synthase gene cluster. The degree of hybridization, which is directly proportional to the transcriptional activity of the gene cluster, was determined (see "Materials and Methods"). Because an equal number of plastids were assayed, the hybridization of each of the stages was normalized to the genome number per plastid at that stage. These results are summarized in Figure 2C. Figure 2C shows that, on an equal gene copy basis, etioplasts transcribe the large ATP synthase gene cluster approximately 3-fold more actively than do the 24-h chloroplasts. When the 24-h chloroplast is compared with the young chloroplast, transcription of this gene cluster increases by a factor of approximately 2. The maturation of the young chloroplast into the mature form is accompanied by a 4-fold decrease in the transcription of this cluster.

DISCUSSION

ATP Synthase-Derived Transcripts during Light-Induced Development

The proportions of total plastid RNAs devoted to transcripts from the two plastid ATP synthase gene clusters appear to be coordinated (Figs. 2E and 4). It is intriguing to speculate that these two clusters, separated by approximately 40 kb, might be coordinately controlled. Further experiments are planned to investigate this possibility.

In contrast to the analysis of the total plastid RNA population, there are only small changes in the levels of the large ATP synthase gene cluster transcripts during light-induced development when normalized to gene dosage. Transcription and RNA stability are the two factors that balance to influence the steady-state levels of these transcripts relative to gene dosage.

Hybridization to probes complementary to each open reading frame plus the atpF intron allowed the effects of light-induced development on both total and specific transcripts from the large ATP synthase gene cluster, including excised introns, to be examined. Although total hybridization to the large ATP synthase gene cluster transcripts normalized to gene dosage varies only slightly between the four developmental stages, the extent of hybridization to individual transcripts within a stage does differ. For example, visual inspection reveals that the 0.5-kb transcript appears to be significantly more abundant in the 24-h compared with the young chloroplast. However, the total hybridization at those two stages differs by only 20%. All of the bands visible in Figure 3 are the result of RNA processing (N. Stollar and M.J. Hollingsworth, unpublished data). From the comparison of individual transcript hybridization to total hybridization, it appears that the extent of RNA processing may vary during the course of light-induced development. Experiments are planned to test this hypothesis.

Transcriptional Activity and RNA Stability of the Large ATP Synthase Gene Cluster during Development

It has been shown that the transcriptional activity of most spinach chloroplast genes remains constant during light-induced development, whereas the steady-state transcript levels of these genes increase significantly, often by as much as 20-fold (3). In contrast, expression of the large ATP synthase gene cluster during development results in a maximum increase of only 2-fold. This observation led to the speculation that this cluster might not be purely under posttranscriptional control, as are many other spinach plastid operons. To investigate this possibility, the transcriptional activity of the large ATP synthase gene cluster was examined on a per

genome basis. The results of this analysis are summarized in Figure 2C. These data, in combination with the northern analysis, were used to deduce the relative stability of the transcripts during light-induced plastid development (Fig. 2D).

The transcriptional activity of this gene cluster undergoes an approximate 3-fold decrease (on a per plastid-genome basis) during the development of etioplast to 24-h chloroplast. However, the quantity of transcripts per genome remains fairly constant (decreasing only by approximately 15%) during this transition. Therefore, the 3-fold decrease in transcription must be balanced by an approximately 3-fold increase in the stability of these transcripts during the etioplast to 24-h chloroplast progression. As the 24-h chloroplast develops to the young stage, there is a 2-fold increase in the transcription of this cluster. This must be balanced by an approximate 2-fold decrease in transcript stability, because the levels of these transcripts per genome only decrease by 20% between these two stages. As the young chloroplast matures, the transcription of this cluster again decreases about 4-fold. This is in contrast to findings from the northern analysis, which revealed that the proportion of these transcripts, on a per genome basis, increases only a small amount (approximately 15%). Therefore, transcripts from this cluster must be approximately 4-fold more stable in mature chloroplasts compared with young chloroplasts. These deduced stability values are summarized in Figure 2D.

Deng and Gruissem (3) have shown that overall chloroplast transcriptional activity peaks in the young chloroplast and is 3- to 4-fold higher at that stage compared with the 24-h stage or mature chloroplast. This is in contrast to the results presented here for the transcription of the large ATP synthase gene cluster, where the highest level of transcriptional activity, on a per genome basis, is found in the etioplasts. A similar situation in which an individual gene or gene cluster is differentially transcribed relative to the overall plastid gene population has also been observed in barley, in which stimulation of psbA transcription in response to illumination was observed to be 2- to 4-fold higher than that observed for rbcL or 16S rRNA genes (15).

It is interesting to note the inverse relationship between the transcriptional activity of this gene cluster and the stability of the transcripts. These two factors balance in a complex fashion throughout plastid development, with the net result that the steady-state levels of the transcripts from this cluster, on a per genome basis, are altered to only a small degree. This is, again, in contrast with the transcription/stability balance for several other spinach plastid gene products. For most of these other genes, transcriptional activity remains relatively constant, whereas the stability of the transcripts steadily increases during the plastid developmental process (3, 4). Obviously, plastids respond to diverse transcription and stability signals. Our data support the hypothesis that plastids may have the ability to regulate the expression of their assorted collection of genes on an individual basis.

SUMMARY

The intricate interaction of transcription and transcript stability determines the proportion of the total plastid RNAs devoted to transcripts from the large ATP synthase gene cluster. A comparison of the effects of these factors on this particular gene cluster to the effects on expression of the plastid genome as a whole reveals that all spinach plastid genes are not regulated in a parallel fashion. Further studies are planned to examine factors that affect the transcription of this gene cluster and compare them to factors influencing the expression of plastid genes in which transcription is controlled differently.

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