

Rubisco, rubisco activase and ribulose-5-phosphate kinase gene expression and polypeptide accumulation in a tobacco mutant defective in chloroplast protein synthesis

Abstract

Expression of the genes for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; *rbcS* and *rbcL*), Rubisco activase (*rca*) and ribulose-5-phosphate (Ru5-P) kinase (*prk*) and accumulation of the polypeptides was examined in chlorophyllous and chlorotic sectors of the D_{P1} mutant of *Nicotiana tabacum*. Plastids from chlorotic sectors of this variegated plastome mutant contained 30S and 50S ribosomal subunits, but had abnormally low levels of plastid polysomes. Consequently, mutant plastids were translationally repressed, unable to synthesize plastid-encoded polypeptides including the large subunit of Rubisco despite the presence of the corresponding mRNAs. Transcripts of *rbcS* accumulated to near wild type levels in chlorotic sectors, but there was little accumulation of the Rubisco small subunit (SS) polypeptide or holoenzyme. Messenger-RNA isolated from chlorotic sectors effectively directed the synthesis of Rubisco SS *in vitro* suggesting that posttranslational factors were responsible for the decrease in Rubisco SS abundance. Transcripts of *rca* and *prk* also accumulated to near wild type levels in chlorotic sectors and a diurnal rhythm in the abundance of *rca* mRNA was detected in green and chlorotic sectors. Despite the low abundance of Rubisco holoenzyme in chlorotic sectors, Rubisco activase and Ru5-P kinase polypeptides accumulated to significant levels. Activities of Rubisco and Ru5-P kinase paralleled protein levels, indicating that active forms of these enzymes were present in chlorotic sectors. The data indicate that the developmental events governing the accumulation of Rubisco activase and Ru5-P kinase polypeptides and the diurnal regulation of *rca* expression were not dependent on the attainment of photosynthetically competent plastids or the accumulation of Rubisco.

Abbreviations: LS – large subunit of Rubisco; Ru5-P kinase – ribulose-5-phosphate kinase; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; SS – small subunit of Rubisco

Introduction

Many of the multimeric complexes present in plastids such as ribosomes, photosynthetic electron transport complexes, and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, E.C. 4.1.1.39) contain both nuclear and chloroplast-encoded polypeptides (Mul-

let 1988). Biosynthesis of these complexes, therefore, requires the coordinate expression of nuclear- and plastid-encoded genes. Light and other environmental signals regulate expression of nuclear and chloroplast-encoded genes (Kuhlemeier et al. 1987). In addition, expression of these genes is coregulated in a cell-specific manner, by developmental signals, and to a

lesser extent endogenous circadian rhythms (Kuhlemeier et al. 1987; Nagy et al. 1988a, b; Giuliano et al. 1988; Taylor 1989b; Rundle and Zielinski 1991a, b; Martino-Catt and Ort 1992; Pilgrim and McClung 1993).

Accumulation of Rubisco, the most abundant of the multimeric plastid complexes, involves not only the coordinate expression of Rubisco LS (plastid-encoded) and SS (nuclear-encoded), but also assembly by nuclear-encoded chaperonins (reviewed by Andrews and Lorimer 1987; Raines et al. 1991; Gatenby and Ellis 1990). Once assembled, Rubisco activity is directly dependent on the activities of two nuclear-encoded chloroplast proteins; Rubisco activase for activation (Salvucci et al. 1985, 1987) and Ru5-P kinase for substrate regeneration (Hurwitz et al. 1956). Studies have shown that the expression of both the *rca* and *prk* genes are mediated by light and developmental signals in a manner which parallels the expression of *rbcS* (Zielinski et al. 1989; Raines et al., 1991). Furthermore, expression of *rca*, like *rbcS*, is regulated by a circadian clock (Rundle and Zielinski 1991b; Pilgrim and McClung 1993; Martino-Catt and Ort 1992). Based on these observations, it has been proposed that regulatory mechanisms may exist to maintain a constant stoichiometry between Rubisco activase and the Rubisco holoenzyme (Zielinski et al. 1989; Rundle and Zielinski 1991b). However, no direct experimental evidence has been presented for the existence of such a mechanism. Furthermore, tobacco transformed with an antisense *rca* gene did not exhibit a reduction of Rubisco protein levels despite a 90% reduction in the protein level of Rubisco activase (Mate et al. 1993; Jiang et al. 1994).

In the present study, we examined the question of coordinate regulation of Rubisco, Rubisco activase and Ru5-P kinase gene expression in a mosaic variegated plastome mutant (D_{P1}) of *Nicotiana tabacum*. The lesion in this plastome mutant causes sections of the leaf, cell layers within a leaf, whole leaves or even entire side branches to be totally achlorophyllous. Here we show that plastids in the chlorotic sectors are defective in the initiation step of translation. Thus, we were able to use the mosaic variegated D_{P1} mutant to study how inhibition of plastid protein synthesis and hence, the accumulation of Rubisco LS, affects the transcription and translation of functionally-related nuclear-encoded photosynthetic genes.

Materials and methods

Plant material

Seeds of the mosaic variegated D_{P1} mutant of *Nicotiana tabacum* were obtained from the USDA-ARS Tobacco Collection Center (Oxford, NC, USA). Seeds were surface sterilized with 70% ethanol (2.5 min) followed by 20% Clorox (15 min). Sterilized seed was germinated at 23 °C on Murashige and Skoog agar media containing 3 g/L sucrose under 100 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ white light. After the first true leaves had developed, mosaic variegated seedlings were transplanted and grown under controlled environmental conditions as previously described (Klein et al. 1993). Unless stated otherwise, plants were approximately 1 meter-in-height when sampled. Chlorotic material was selected from leaves that were achlorophyllous over at least half of the lamina.

Plastid isolation and plastid number

To reduce the starch content of green leaf sectors, tobacco plants were maintained in the dark for 24 h and then illuminated for 2 h prior to plastid isolation. Plastids were isolated from chlorotic leaf sectors (Baumgartner et al. 1989) with several modifications. Homogenates from chlorotic leaf sectors were centrifuged for 5 min at 10,000 g, layered on Percoll step gradients (upper layer 30% Percoll, lower layer 65% Percoll) and centrifuged at 5,000 g for 10 min. Intact plastids were collected from the Percoll interface and concentrated by centrifugation as described (Baumgartner et al. 1989). Plastids from green leaf sectors were isolated as described (Mullet et al. 1986). Plastids isolated from green and chlorotic leaf sectors were counted using a hemacytometer and a microscope equipped with a 20X phase-contrast lens. In one experiment, plastids were fractionated into membrane and stromal phases; membrane-associated proteins were resolved by SDS-PAGE and silver stained as previously described (Klein and Mullet 1986, 1987).

Preparation of polysomes and analysis on sucrose density gradients

Polysomes were prepared from intact plastids (9.0×10^7) as described (Klein et al. 1988) except that sucrose gradients were centrifuged for 2.5 h at 260,000 g. After centrifugation, sucrose gradients were fractionated and the contents monitored for absorbance at 260 nm.

Northern analysis

RNA from intact plastids was isolated by phenol extraction as described (Klein and Mullet 1986). Total RNA from leaf tissue was cesium-trifluoroacetate-purified (Pharmacia-LKB,¹ Piscataway, NJ, USA) as described (Klein et al. 1993). Polyadenylated mRNA was isolated from total RNA samples utilizing the Dynabeads Oligo (dt)₂₅ mRNA purification kit (Dyna, Inc. Lake Success, NY, USA). Polyadenylated mRNA (0.5 µg per lane) or total RNA (2 µg per lane) was loaded on formaldehyde gels (Sambrook et al. 1989) and transferred to GeneScreen nylon membranes (DuPont-NEN, Wilmington, DE, USA). Conditions for prehybridization and hybridization with radiolabeled antisense-RNA probes were as described (Klein et al. 1993). The antisense probe for *rbcL*, *psbA*, and *psaA-B* have been described (Klein and Mullet 1990). The northern probe for *rca* was a 1300-b antisense RNA from tobacco (Salvucci and Klein 1994); the probe for *prk* was a 1500-b antisense RNA from ice plant (Michalowski et al. 1992); the probe for *rbcS* was a 743-b antisense RNA from tobacco (Klein and Salvucci 1992). Northern blots were scanned with an image acquisition densitometer (BioImage, Milligen/Biosearch, Ann Arbor, MI, USA) to determine the relative intensity of the mRNA signal which was quantified on the basis of whole-band analysis.

In vitro translation of polyA mRNA

Polyadenylated mRNA isolated from green and chlorotic sectors was translated in a wheat germ system in the presence of [³⁵S]Met as detailed by the manufacturer (Promega Inc., Madison, WI, USA). Samples were electrophoresed in 7.5–15% gradient SDS-PAGE and radiolabeled polypeptides were visualized by fluorography (Klein and Salvucci 1992).

Leaf extraction, SDS-PAGE, Western blot analysis and enzyme activities

Leaf discs from green and chlorotic sectors were extracted in 100 mM Tricine, pH 8.0, 5 mM MgCl₂, 1 mM EDTA, 10 mM dithiothreitol, 1% (w/v) polyvinylpyrrolidone-40, 1 mM phenylmethylsulfonate and 10 µM leupeptin (Buffer 1). Aliquots

of the crude homogenates were taken for determination of chlorophyll (Arnon 1949) or were added to boiling SDS in preparation for Western blot analysis (Crafts-Brandner et al. 1990). The remainder of the homogenate was centrifuged for 1 min at 13,000 g and aliquots of the supernatant fraction electrophoresed in 7.5–15% gradient SDS-PAGE gels to determine polypeptide profiles (Klein and Salvucci 1992). Aliquots of the supernatants were also used for determination of total soluble protein (Bradford 1976), Rubisco activity, and Ru5-P kinase activity at 30 °C (Crafts-Brandner et al. 1990). Two to three separate samples were used for each determination and each sample was assayed in duplicate.

For Western blots analysis, polypeptides from total leaf extracts were electrophoresed in 12% SDS-PAGE mini-gels and electrophoretically transfer to PVDF membranes (Salvucci et al. 1993). Blots were probed with polyclonal antibodies to tobacco Rubisco, Ru5-P kinase (Crafts-Brandner et al. 1990) and Rubisco activase (Salvucci et al. 1993). Immunogenic polypeptides on the blots were visualized using an alkaline phosphatase secondary antibody system. Polypeptide levels were quantified by densitometry as described above. Known amounts of purified tobacco Rubisco, Rubisco activase and Ru5-P kinase that spanned the concentration range of the samples were electrophoresed on each gel to serve as standards for quantitation.

In vivo protein synthesis

Pulse labeling experiments were performed on leaf segments from green and chlorotic sectors of the D_{P1} mutant. Following removal of the lower epidermis, segments were floated on a solution of 0.5 M sorbitol and 20 mM MES-KOH, pH 6.5, containing 1 mCi carrier-free [³⁵S]Met and either 50 µg/ml cycloheximide or an equal volume of buffer. Segments were irradiated with 350 µmol photons·m⁻²·s⁻¹ of white light at 25 °C during the 15 min incubation and then frozen in liquid N₂. The frozen leaf material was extracted in Buffer 1 (described above) and centrifuged for 5 min at 13,000 g to separate soluble and insoluble material. Following resuspension of the pelleted material, acetone was added to aliquots of the soluble and insoluble (pelleted) fractions to 80% (v/v) and incubated 1 h at -20 °C. Precipitated protein was collected by centrifugation, resuspended in SDS-sample buffer (Crafts-Brandner et al. 1993) and electrophoresed in 7.5–15% gradient SDS-PAGE. Radiolabeled polypep-

¹ Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

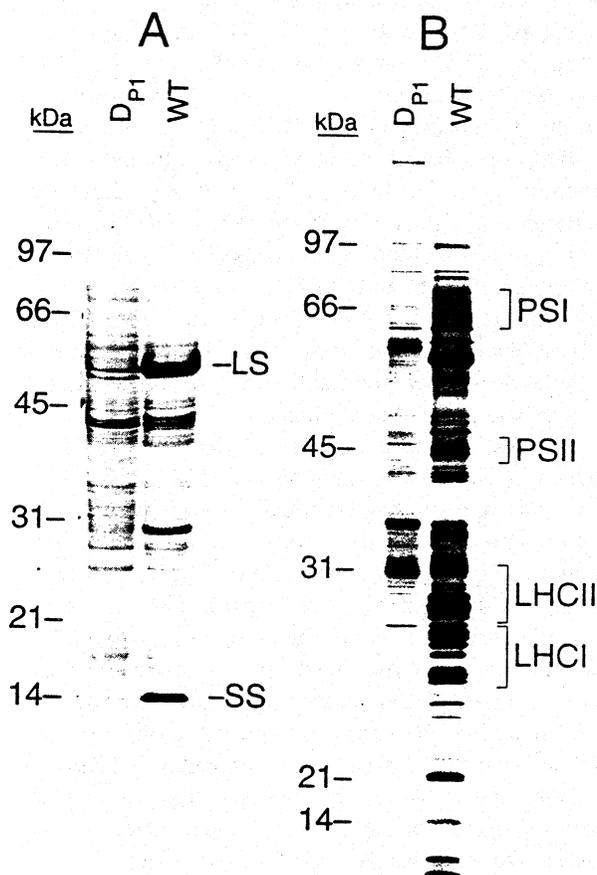


Fig. 1. Accumulation of polypeptides in D_{P1} and wild type tobacco tissues. Panel A) Total soluble leaf protein from chlorotic (D_{P1}) and green (WT) sectors was resolved by 7.5–15% SDS-PAGE and stained with Coomassie blue. Samples were loaded on an equal protein basis. Panel B) Plastids were isolated from D_{P1} and wild type tissues and a membrane-enriched fraction obtained. Membrane-associated polypeptides were resolved by 7.5–15% SDS-PAGE and silver stained. Samples in panel B were loaded on an equal plastid number basis (1.2×10^6 plastid equivalents per lane). Identities of protein bands were assigned based on migration versus that of MW standards.

tides were separated by SDS-PAGE and visualized by fluorography (Klein and Salvucci 1992).

Results

Protein, transcript, and plastid polysome content in white and green sectors of the D_{P1} mutant

Several lines of evidence from the initial histogenic and genetic analysis indicated that the lesion in the

chlorophyll variegated tobacco mutant, D_{P1} , resides in the plastid genome (Burk et al. 1964). The evidence includes, maternal inheritance of the D_{P1} phenotype, nonrandom clustering of white and green tissues, and the presence of both green and achlorophyllous plastids in cells of young, but not mature leaves. In the present study, examination of the polypeptide composition of chlorotic leaf sectors revealed a marked reduction in the accumulation of several chloroplast-localized proteins (Fig. 1). These proteins included the Rubisco LS and SS (Fig. 1A) as well as thylakoid membrane proteins such as the chloroplast-encoded chl *a*-apoproteins of PSI and PSII and the nuclear-encoded chl *a/b*-apoproteins (LHCI, LHCII) (Fig. 1B).

Transcript levels of *rbcl*, *psbA*, and *psaA-B* in D_{P1} mutant plastids are shown in Fig. 2. When normalized on the basis of plastid number, the levels of *psbA* and *psaA-B* transcripts were similar in wild type (green) and mutant (chlorotic) plastids. In contrast, the level of *rbcl* mRNA was approximately 3-fold higher in mutant plastids. These results indicate that the lesion in D_{P1} plastids did not lead to a global disruption of plastid transcriptional activity.

Having established that the lesion in D_{P1} plastids was not at the level of chloroplast transcription or transcript accumulation, we examined the patterns of protein synthesis in green and chlorotic sectors of the D_{P1} mutant. When wild type leaf sectors were pulse labeled *in vivo* with [35 S]Met, radiolabel was incorporated into a host of nuclear-encoded leaf proteins (data not shown). To better examine the synthesis of organelle-encoded polypeptides, green and chlorotic leaf sectors were pulse labeled in the presence of cycloheximide, an inhibitor of cytoplasmic protein synthesis (Fig. 3). Wild type leaf sectors radiolabeled several chloroplast-encoded polypeptides including Rubisco LS and several identifiable membrane-associated polypeptides such as the D polypeptides (product of the *psbA* and *psbD* genes) and PSII (product of the *psbB* and *psbC* genes). In contrast, radiolabel was not incorporated into chloroplast-encoded polypeptides when chlorotic sectors of the D_{P1} mutant were pulse labeled with [35 S]Met under similar conditions. Since short term treatment with cycloheximide does not markedly alter chloroplast protein synthesis (Klein and Mullet 1986), these results indicated that the D_{P1} plastids were translationally repressed.

Examination of polysomes in plastids from chlorotic sectors indicated that the block in plastid protein synthesis was probably at the level of translation initiation. Sucrose gradient profiles of polysomes from wild type

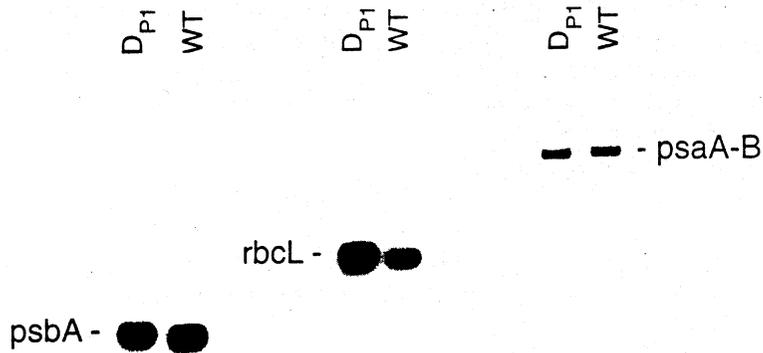


Fig. 2. Northern blot analysis of transcripts in D_{P1} and wild type tobacco plastids. Messenger-RNA from D_{P1} (chlorotic) and wild type (green) plastids was loaded on an equal plastid number basis (1.2×10^6 plastid equivalents per lane). Northern blots of *psbA*, *rbcL* and *psaA-B* were exposed to X-ray film for 1, 2, and 12 h, respectively.

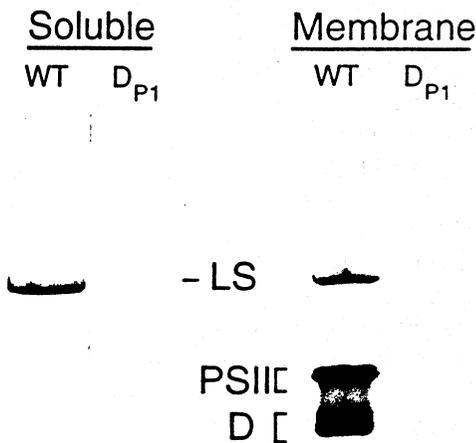


Fig. 3. *In vivo* labeled plastid polypeptides from D_{P1} and wild type tobacco tissues. Leaf segments were pulse-labeled for 15 min with [35 S]Met in the presence of 50 μ g/ml cycloheximide. Homogenates prepared from the tissue were fractionated into soluble and membrane-associated proteins and samples containing equal amounts of protein were resolved by 7.5–15% SDS-PAGE. SDS-PAGE gels were flouorographed and exposed to X-ray film. Identities of protein bands were assigned based on migration versus that of MW standards.

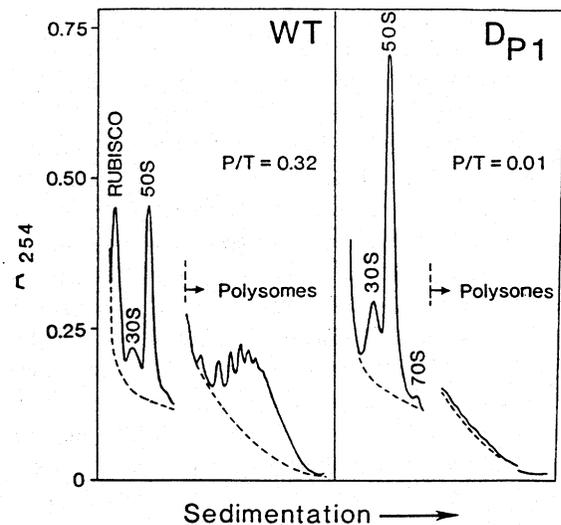


Fig. 4. Polysome profiles from D_{P1} and wild type tobacco plastids. P/T values represent the proportion of total ribosomes (T) aggregated into polysomes (P). The vertical dashed lines dividing polysomal and nonpolysomal fractions indicate a 5-fold increase in scan sensitivity for polysomal fractions. Dashed lines below polysome profiles represent absorbance baseline generated by sedimenting post-ribosomal supernatants.

plastids showed that a considerable portion of 70S ribosomes were in polysomes (Fig. 4). In contrast, similar profiles from D_{P1} plastids indicated abnormally low numbers of polysomes. D_{P1} plastids, however, were not ribosome-deficient as evidenced by the presence

of 30S and 50S ribosomal subunits. The proportion of unassembled ribosomal subunits was significantly greater for mutant plastids as evidenced by the low P/T ratios (proportion of total ribosomes aggregated into polysomes). The above results support the assertion that the D_{P1} plastome mutant was blocked in plastid protein synthesis because of a translational defect at the level of initiation.

Expression of nuclear genes encoding plastid proteins in D_{P1} mutant

Since D_{P1} plastids are defective in chloroplast translation and hence, Rubisco LS synthesis, this mutant would be well suited for examining the question of coordinate regulation of Rubisco LS and SS, Rubisco activase and Ru5-P kinase gene expression and polypeptide accumulation. The accumulation of transcripts of the nuclear genes *rbcS*, *rca*, and *prk* during the course of D_{P1} leaf development is shown in Fig. 5A. At the earliest stage of leaf development examined (LAI = 7 cm²), transcripts of *rbcS*, *rca*, and *prk* accumulated in chlorotic leaf sectors. The steady state levels of *rbcS*, *rca* and *prk* mRNA in chlorotic sectors generally paralleled the levels observed in green tissue although the decline in transcript levels with leaf age was not as pronounced in chlorotic sectors. The accumulation of *rbcL* transcripts increased with development in both wild type and D_{P1} mutant tissues. When expressed on an equal total RNA basis, *rbcL* transcript levels were lower in D_{P1} mutant tissues which likely reflects the lower number of plastids per unit leaf area in chlorotic sectors (Burk et al. 1964). Ethidium bromide-stained agarose gels revealed that plastid rRNA accumulated to similar levels in green and chlorotic sectors (data not shown). It should be noted that quantitative differences in transcript levels between green and chlorotic D_{P1} sectors (and the fluctuation of *prk* and *rca* transcript in chlorotic sectors) may also reflect developmental differences between green and chlorotic sectors of the leaf. Nevertheless, Northern analyses demonstrate that the nuclear genes of *rbcS*, *rca* and *prk* are transcribed in D_{P1} mutant sectors.

Poly(A) mRNA isolated from D_{P1} chlorotic tissues directed the synthesis of the Rubisco SS polypeptide in an in vitro wheat germ translation system (Fig. 5B). In fact, the profiles of in vitro synthesized polypeptides were very similar for poly(A) mRNA isolated from mutant and wild type leaf sectors. The level of in vitro synthesized Rubisco SS was slightly greater

for poly(A) mRNA isolated from green tissues compared to chlorotic tissue. However, compared to the differences in the levels of Rubisco SS polypeptide in green and chlorotic tissue (Fig. 1 and below) the differences were comparatively small. Thus, the marked reduction in Rubisco SS polypeptide levels in chlorotic sectors cannot be ascribed entirely to differences in *rbcS* mRNA translatability.

Protein levels and enzyme activity of nuclear-encoded chloroplast proteins in D_{P1} mutant

On a leaf area basis, total soluble protein levels in chlorotic sectors of the D_{P1} mutant were 40 to 50% lower than in the green sectors (Table 1). Western blot analysis showed that the polypeptide levels corresponding to Rubisco (LS and SS), Rubisco activase (42 kDa), and Ru5-P kinase (43 kDa) were also reduced in chlorotic sectors. While the extent that each protein was reduced in chlorotic sectors depended on the stage of leaf development, the reduction in the level of Rubisco was consistently greater than that of Rubisco activase and Ru5-P kinase. In expanding leaves, the amount of Rubisco LS and SS in chlorotic sectors was below the limit for quantitation. Rubisco subunit levels in chlorotic sectors were estimated at less than 6% of the level in green sectors on a leaf area basis or less than 14% on the basis of total soluble leaf protein. Chlorotic sectors contained comparatively higher levels of Rubisco activase and Ru5-P kinase equivalent to 24% of the green sector level on an area basis or 49 and 60%, respectively, on the basis of total soluble protein (Table 1, see also Fig. 6). In mature, fully-expanded leaves, the levels of Rubisco activase and Ru5-P kinase (per unit leaf area) were similar in chlorotic and green sectors. When expressed on a soluble leaf protein basis, Rubisco activase and Ru5-P kinase levels were actually higher in the chlorotic sectors of mature leaves. By comparison, the level of Rubisco polypeptides in chlorotic sectors was still below the limits for quantitation. In an attempt to estimate the decrease of Rubisco protein levels in mature chlorotic sectors, dilution series of wild type and chlorotic protein extracts were analyzed by Western blot analysis (data not shown). When expressed on an equal protein basis, the level of Rubisco protein of chlorotic sectors was less than 10% of the level in green sectors of mature leaves.

The activities of Rubisco and Ru5-P kinase in crude extracts prepared from chlorotic tissue generally paralleled the level of protein estimated by Western blot analysis particularly in expanding leaves (Table 1).

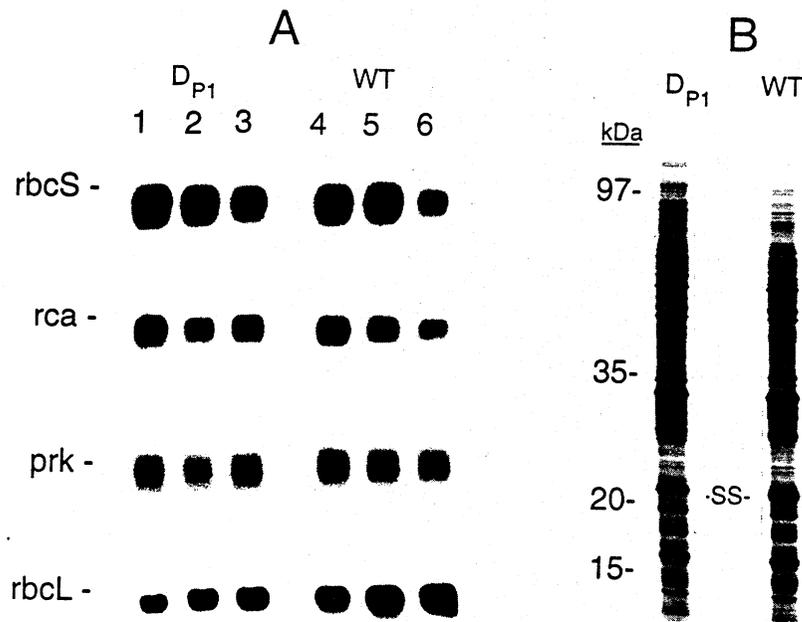


Fig. 5. Panel A) Northern blot analysis of mRNA isolated from D_{P1} and wild type leaf sectors. RNA was isolated from chlorotic (D_{P1}) and green (WT) leaves of approximately 7 cm² (lanes 1, 4), 170 cm² (lanes 2, 5), or 570 cm² (lanes 3, 6). Northern blots were loaded on an equal RNA basis. Blots of *rbcS*, *rca*, *prk* and *rbcL* were exposed to X-ray film for 1, 3, 5, and 1 h, respectively. Each Northern blot was repeated at least twice and a representative blot is shown. Panel B) Autoradiograph of in vitro translation products of poly(A) mRNA from D_{P1} and wild type tobacco tissues. In vitro labeled polypeptides were resolved by 7.5–15% SDS-PAGE, flouorographed, and exposed to X-ray film for 6 h. The identity of Rubisco SS was confirmed by Western blot analysis.

Table 1. Chlorophyll, soluble protein, and the amounts and activities of Rubisco, Rubisco activase and Ru5-P kinase in green (wild type) and chlorotic (D_{P1}) leaf sectors from expanding (LAI = 175 cm²) and mature (LAI = 530 cm²) leaves of the D_{P1} mutant

Parameter ^a	LAI = 175 cm ²		LAI = 530 cm ²	
	Wild type	D_{P1}	Wild type	D_{P1}
Chlorophyll (mg·cm ⁻²)	0.040	0.00012	0.030	0.00034
Protein (mg·cm ⁻²)	0.797	0.392	0.240	0.146
Rubisco (mg·cm ⁻²)	0.162	< 0.011 ^b	0.045	< 0.011
Rubisco activase (μg·cm ⁻²)	3.90	0.90	0.91	0.85
Ru5-P kinase (μg·cm ⁻²)	1.20	0.30	0.40	0.47
Rubisco activity (U·cm ⁻²) ^c	0.314	0.012	0.088	0.006
Ru5-P kinase activity (U·cm ⁻²) ^d	1.50	0.38	0.73	0.29

^aStandard errors were within 10% of the reported values.

^bRepresents the lower limit for quantitation.

^cActivity of fully carbamylated enzyme, measured after incubation for 10 min at 25 °C with 10 mM NaHCO₃.

^dActivity of the fully reduced enzyme, measured after incubation for 10 min at 25 °C with 10 mM dithiothreitol.

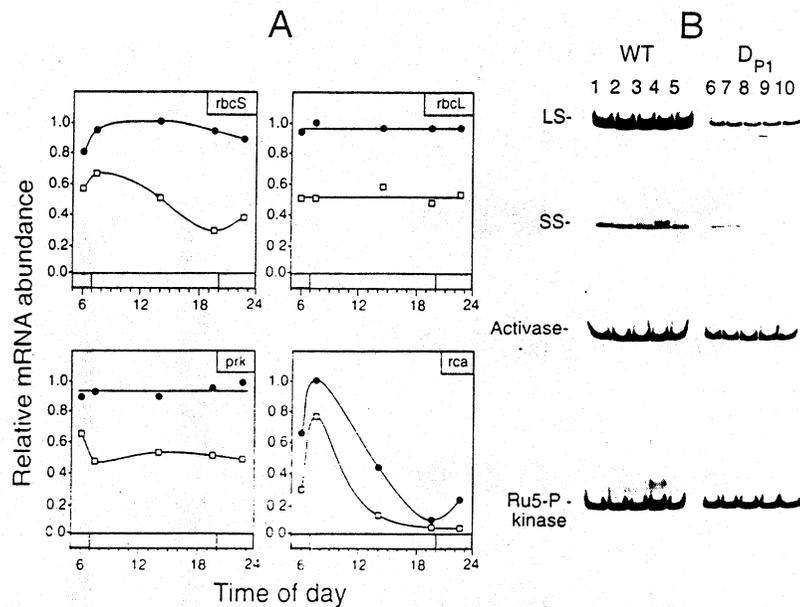


Fig. 6. Diurnal rhythm in the accumulation of proteins and transcripts in DP_1 and wild type tobacco tissues from plants exposed to a 13 h light/11 h dark photoperiod. The light period was from 7:00 to 20:00. Panel A) RNA was isolated from tissues harvested at various times throughout the photoperiod and Northern blots conducted. Quantitation of the resulting autoradiographs was conducted by scanning densitometry. Relative mRNA abundance values were calculated by setting the maximum densitometry value to 1.0 for each gene. Relative mRNA abundance values for wild type tissues are represented by closed circles and values for DP_1 tissues by open squares. Each data point represents duplicate observations from replicated Northern blots. Panel B) Western blot analysis of Rubisco LS and SS, Rubisco activase and Ru5-P kinase was conducted on chlorotic and green tissue from the DP_1 mutant harvested at 6:00 (lanes 1, 6), 7:30 (lanes 2, 7), 14:00 (lanes 3, 8), 19:30 (lanes 4, 9) and 22:30 h (lanes 5, 10). Protein was extracted from equal areas of tissue and the samples were electrophoresed in each lane without adjustment.

When expressed on an area basis, Rubisco activity in the chlorotic sectors of expanding and mature leaves was 4 and 7% of the activity in green sectors, respectively. When expressed on a leaf area basis, Ru5-P kinase activity was 26 and 40% of the green sector activity in expanding and mature leaves, respectively. On the basis of total soluble protein, this represents only a 48% reduction in Ru5-P kinase activity in chlorotic sectors of expanding leaves and a 35% reduction in activity in mature leaves. Assay of Rubisco activase activity was not possible in crude leaf extracts from tobacco. However, the Rubisco activase protein present in DP_1 chlorotic sectors (see Fig. 6) was catalytically competent following partial purification (data not shown).

Diurnal rhythm of transcript accumulation in DP_1 chlorotic sectors

It has been previously established that circadian rhythms in *rca* transcript levels exist in green leaf tissue of several plant species (Rundle and Zielins-

ki 1991b; Martino-Catt and Ort 1992; Pilgrim and McClung 1993). To determine whether *rca* transcript accumulation in tobacco exhibits a characteristic diurnal pattern and whether such a rhythm was apparent in chlorotic sectors of the DP_1 mutant, wild type and mutant sectors from expanding leaves were harvested at various times during the photoperiod and protein and mRNA levels were quantified (Fig. 6). A modest diurnal rhythm in *rbcS* transcript accumulation was apparent in chlorotic leaf sectors, while significant diurnal rhythms in *prk* or *rbcL* transcript accumulation were not observed (Fig. 6A). Of the nuclear and chloroplast genes examined, only *rca* exhibited a marked diurnal rhythm in transcript accumulation. The pattern of *rca* transcript accumulation was similar in green and chlorotic tissues with an apparent maximum at the beginning of the light period. By the mid point of the light period, *rca* transcripts in wild type and mutant tissues had begun to decline precipitously. Interestingly, despite the marked diurnal fluctuation in *rca* transcript, protein levels of Rubisco activase remained constant throughout the photoperiod (Fig. 6B).

Discussion

Mutations that interrupt specific processes in the chloroplast provide powerful tools for examining the coordinate expression of plastid- and nuclear-encoded genes of chloroplast proteins (reviewed by Somerville 1986; Taylor 1989a). In the present study, we characterized the molecular basis for the photosynthetic defect in the plastome mutant of tobacco, D_{P1} . Having determined the molecular defect in plastid gene expression, we utilized the D_{P1} mutant phenotype to examine the coordinate regulation of Rubisco LS gene expression with that of functionally-related, nuclear-encoded proteins including Rubisco SS, Rubisco activase and Ru5-P kinase.

Chlorotic sectors of the D_{P1} mutant contained plastids that are translationally repressed and hence, deficient in the synthesis of plastid-encoded proteins including Rubisco LS. Several lines of evidence presented here indicate that the repression of plastid translation in the D_{P1} mutant is caused by a disruption in translation initiation. The steady-state levels of chloroplast-encoded polypeptides were markedly reduced though full-length transcripts for several plastid-encoded genes accumulated in chlorotic sectors. Sucrose gradient profiles of plastid polysomes showed abnormally low levels of polysomes in chlorotic sectors despite the presence of 30S and 50S ribosomal subunits. This, combined with the lack of radiolabel incorporation into chloroplast-encoded proteins under pulse labeling conditions revealed a molecular basis for the D_{P1} phenotype likely resides in a defect in initiation of plastid translation.

At present, it is unknown where the primary genetic defect resides in the genome of D_{P1} plastids. Plastid-encoded gene products that are involved in the chloroplast translation machinery include ribosomal and transfer RNA, ribosomal protein operons, and open reading frames homologous with *E. coli infA* and *secX* genes (Shinozaki et al. 1986). Mutations in any number of these open reading frames could potentially alter the functionality of the ribosomal subunits leading to a repression in plastid translation. Recently, Barkan (1993) characterized several nuclear mutants of maize which, like the D_{P1} mutant, exhibited defects in chloroplast polysome assembly. Plastids of the *hcf7* mutant were defective in 16S rRNA processing, which caused a global reduction in plastid translation. Further studies are necessary to determine the primary genetic defect in the genome of D_{P1} plastids.

It should be noted that the D_{P1} phenotype is slightly leaky as evidenced by the low but detectable level of polysomes and Rubisco protein in these tissues. Further, since one of the plastid RNA polymerases contains several plastid-encoded subunits (Mullet 1993), it is reasonable to suspect that the D_{P1} mutant is capable of a minimal level of protein synthesis. Nevertheless, the greatly reduced level of Rubisco LS synthesis in D_{P1} plastids made this photosynthetic mutant a useful experimental tool for analyzing the interaction of the nuclear and plastid genomes for the synthesis of functionally related proteins. This approach has been extensively exploited in *Chlamydomonas* where analysis of mutants has proven to be a powerful approach in understanding the assembly of chloroplast protein complexes (reviewed by Rochaix 1992).

Examination of *rbcS* gene expression and polypeptide accumulation in chlorotic sectors deficient in Rubisco holoenzyme suggested that posttranslational mechanisms maintain a constant stoichiometry between Rubisco LS and SS. Chlorotic sectors accumulated near wild type levels of *rbcS* mRNA though accumulation of the 14 kDa Rubisco SS polypeptide was markedly reduced. Further, mRNA isolated from chlorotic sectors effectively directed the synthesis of Rubisco SS in vitro demonstrating that the control of Rubisco SS accumulation was exerted at a posttranslational level. These observations are in accordance with the widely held hypothesis that when either Rubisco subunit type is synthesized in excess, the other subunit type is rapidly proteolyzed within the plastid to maintain a set stoichiometry for holoenzyme production (Spreitzer et al. 1985; Schmidt and Mishkind 1983; Rodermeier et al. 1988). Unable to assemble with Rubisco LS, imported Rubisco SS is apparently recognized in D_{P1} plastids as having an abnormal conformation and hence, is targeted for degradation.

Examination of transcript levels of *rca* and *prk* in chlorotic leaf sectors revealed that, like *rbcS*, these photosynthesis-related genes are actively transcribed in chlorotic leaf sectors. Previous studies have shown that expression of many photosynthesis-related genes appears to be under a strict transcriptional program during the course of leaf development and plastid biogenesis (Raines et al. 1991). During the course of normal leaf development, this transcriptional program would govern the accumulation of photosynthetic enzymes as young leaves develop functional chloroplasts (Kuhlemeier et al. 1987; Gilmartin et al. 1990).

While light- and developmental-dependent signals have been shown to govern the expression of *rca* and

prk (Raines et al. 1989, 1991; Zielinski et al. 1989; Rundle and Zielinski 1991b; Michalowski et al. 1991), our results show that these developmental signals are not closely coordinated with accumulation of Rubisco or related to the photosynthetic activity of the chloroplast. Active forms of Ru5-P kinase and Rubisco activase accumulated in excess of Rubisco holoenzyme in D_{P_1} mutant plastids. Hence, stoichiometric ratios between Rubisco, Rubisco activase and Ru5-P kinase were not maintained as has been previously proposed. The results of Mate et al. (1993) and Jiang et al. (1994) utilizing tobacco transformed with an antisense *rca* gene construct further substantiates the view that expression and accumulation of Rubisco activase is not tightly coordinated with that of Rubisco holoenzyme. Post-transcriptional mechanisms did not operate to maintain Ru5-P kinase and Rubisco activase in stoichiometric ratios with Rubisco in plastids where protein synthesis was disrupted. The accumulation of Rubisco activase and Ru5-P kinase in D_{P_1} mutant plastids may reflect the fact that neither of these proteins have been shown to form a long-lived complex with Rubisco holoenzyme. The analysis of mutants defective in PS II and PS I photoactivity has revealed that when a lesion causes the loss of a core component of a complex, rapid degradation of other subunits of the complex is observed (Rochaix 1992). In contrast, proteins that are not subunits of the complex (but are functionally related) often accumulate to wild type levels. As a result, unassembled Rubisco SS polypeptides are proteolyzed in D_{P_1} plastids, while Rubisco activase and Ru5-P kinase accumulate to near wild type levels.

In addition to the developmental and tissue-specific signals governing the expression of nuclear genes encoding plastid proteins, a subset of these genes including *rca* and *rbcS* have been shown to be controlled by a circadian clock (Rundle and Zielinski 1991; Martino-Catt and Ort 1992; Watillon et al. 1993; Pilgrim and McClung 1993). In the present study we extend these observations to tobacco by demonstrating the presence of a strong diurnal rhythm in *rca* transcript accumulation. We further show that the diurnal rhythm in *rca* transcript accumulation was similar in green and D_{P_1} chlorotic sectors. These results support previous observations with plastid ribosome-deficient barley seedlings where the circadian rhythm in the abundance of *cab* transcripts was detectable despite the low absolute levels of *cab* mRNA (Hess et al. 1994). Hence, the cellular mechanisms used to interface gene expression of *rca* with the circadian clock

are not dependent on functional plastid translational apparatus or the photosynthetic activity of the tissue. It should be noted, however, that a physiological role for a circadian rhythm in *rca* transcript accumulation is unclear since the level of Rubisco activase in mature tobacco leaves does not exhibit a similar oscillation. It has been proposed (Marino-Catt and Ort 1992; Pilgrim and McClung 1993) that a physiological role in photosynthetic performance may exist in early stages of leaf development where Rubisco activase levels are low and a circadian rhythm in *rca* transcript abundance could impact protein levels. Closer examination of leaf tissue in the early stages of photosynthetic development may elucidate whether regulation of Rubisco activity is governed by the circadian oscillation in *rca* gene expression.

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