Differential Expression of the Ribulose Bisphosphate Carboxylase Large Subunit Gene in Bundle Sheath and Mesophyll Cells of Developing Maize Leaves Is Influenced by Light

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ABSTRACT

We have investigated the influence of light on the mRNA of the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase in mesophyll and bundle sheath cells of the C₄ plant Zea mays. The gene is transcribed in both cell types in leaves of seedlings grown in the dark. However, upon illumination, the level of mRNA declines and becomes undetectable in mesophyll cells after 72 hours. On the contrary, in bundle sheath cells the two transcripts of the same gene increase to peak after 24 hours of greening and then decrease to a steady state level. This study was made possible by the development of improved methods for the isolation of two maize leaf cell types suitable for the extraction of high quality RNAs at different developmental states, i.e. etiolated, greening and green.

Transcripts of six regions comprising about 19% of the maize chloroplast chromosome increase in abundance during light-induced maturation of etioplasts in dark-grown seedlings into photosynthetically competent chloroplasts (11). One of these regions carries the genes for RuBPCase LS, as well as the fused DNA sequences coding for the β-ε CF₁. The levels of transcripts of these CF₁ and RuBPCase genes accumulate to a maximum level after about 20 h of greening and then drop in abundance (4, 10–12). However, maize is a C₄ plant thus photosynthetic carbon fixation is the result of the coordinated activities of MC and BSC. In mature green leaves of maize, the mRNA for the β-ε CF₁ is abundant in chloroplasts of both MC and BSC, but that for the large subunit of RuBPCase is present only in the latter cell type (8, 10). Accordingly, it is intriguing to determine when mesophyll and bundle sheath plastids differentiate. How is the transcription of the RuBPCase LS gene regulated in MC?

To understand the molecular developmental biology of C₄ leaves and the regulation of expression of the RuBPCase LS gene, we have developed methods for preparing high quality RNA from well separated MC and BSS of etiolated and greening leaves of maize seedlings. Mesophyll etioplasts contain RuBPCase LS mRNA but the level of these transcripts falls rapidly during light-induced maturation of the plastids. The expression of RuBPCase LS gene is strikingly different in bundle sheath plastids.

MATERIALS AND METHODS

Plant Material and Growth. Maize seedlings (Zea mays: FR9 cms x FR37; Illinois Foundation Seeds) were grown in vermiculite at 28°C either in a dark room for 7 d or in a greenhouse under incandescent light of 1400 lux on a 16 h photoperiod for 10 d. Seven-day-old etiolated seedlings were greened by illumination with incandescent light of 1400 lux at 28°C.

Isolation of Bundle Sheath Strands. Three g of middle sections of expanded leaves were cut into 2 × 2 mm squares with a sharp razor blade. The leaf pieces were put into a small plastic beaker containing 25 ml of disruption buffer (50 mm Tris-HCl pH 8.0, 0.6 m sorbitol, 1 mm MgCl₂, 10 mm vanadyl ribonucleoside complex, 100 mm β-mercaptoethanol). The tissue was disrupted with a Polytron (the beaker was set in an ice water bath) beginning at speed 3 for 5 s to mix the buffer and leaf pieces, then at speed 6 for 40 s. The foam formed during the grinding was removed with Kimwipes before filtration though an 80 μm nylon mesh net. The residue was resuspended in another 25 ml of disruption buffer and the same procedure was followed twice more. At the end, the pure BSS retained by the 80 μm net were rinsed with another 25 ml of disruption buffer, blotted dry between paper towels, and then dropped into liquid N₂ and stored at −70°C (5–7, 9).

Isolation of Mesophyll Protoplasts. Using a fresh sharp razor blade, 5 g of the middle sections of leaves were cut perpendicularly to the midrib to give 0.5 to 1 mm strips. Leaf strips were added to 80 ml of enzyme buffer (20 mm Mes [pH 5.5], 1 mm MgCl₂, 0.6 mm sorbitol, 2% [w/v] cellulase [Onozuka SS; All Japan Biochemicals], 0.1% [w/v] macerase [Calbiochem]) in a flask with a side arm; suction was applied until all the leaf strips were infiltrated. The mixture was then poured into a 150 × 15 mm Petri dish and the digestion continued for 3 to 5 h at room temperature. At the end of the digestion the enzyme buffer and broken cells were discarded by filtration through a 135 mesh...
nylon net. The residual partly digested leaf segments were resuspended in 50 ml washing buffer (50 mM Tris-HCl [pH 7.5], 0.6 M sorbitol, 1 mM MgCl₂, 10 mM vanadyl ribonucleoside complex, 100 mM β-mercaptoethanol) in the same Petri dish. Leaf strips were pressed gently with a spatula to release the MP which were then separated by filtration through an 80 μm net. Protoplasts were collected by centrifugation at 300g for 5 min, the pellet was resuspended gently and washed with 25 ml washing buffer. Finally, the MP were concentrated by recentrifugation and were resuspended in 2 ml of washing buffer, dropped into liquid N₂, and stored at −70°C (5–7, 9).

**RNA Extraction.** The frozen BSS and MP were ground to a fine powder in liquid N₂ with a mortar and pestle. Fifteen ml lysis buffer (100 mM Tris-HCl, pH 8.6, 2% [w/v] Sarkosyl, 4 mM guanidium thiocyanate, 25 mM EDTA, 25 mM EGTA, 100 mM β-mercaptoethanol) and 7.5 ml phenol were added per MP preparation from 5 g of leaf tissue cells (yield about 500 μg RNA) or BSS prepared from 3 g of leaf tissue (yield 150 μg RNA). The slurry was poured into a 30 ml polypropylene tube, shaken vigorously, then 7.5 ml of chloroform:isoamyl alcohol (24:1, v/v) was added and mixed well. The tubes were spun in a Sorvall SS-34 rotor at 8 K rpm for 4°C for 5 min. The supernatant was removed taking special care not to carry along any interphase and extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). The nucleic acids were precipitated by adding 0.6 volume of isopropanol to the supernatant and freezing at −70°C for at least 1 h or at −20°C overnight. The RNA pellets were rinsed with 95% ethanol, vacuum-dried, resuspended in sterile deionized distilled H₂O, and stored at −70°C (2). Poly A⁺ mRNA was separated from total RNA by chromatography on an oligo(dT) cellulose (P. L. Biochem) column as described (1) except that LiCl was used instead of NaCl to prevent precipitation of the SDS.

**RNA Electrophoresis and Analysis.** Total RNA samples of 30 to 60 μg were denatured in 50% (v/v) formamide, 2.2 M formaldehyde, 1× MOPS buffer at 65°C for 5 min and applied to a 1.2% (w/v) agarose gel with 0.2 M formaldehyde for fractionation. After electrophoresis was completed, the gel was stained with 1 μg/ml ethidium bromide for 30 min and destained in distilled H₂O for another 30 min for visualization of RNA. The gel was then soaked for 30 min in an alkaline solution (50 mM NaOH, 10 mM NaCl) to hydrolyze the RNA partially to improve the efficiency of transfer to GeneScreen (NEN) or nitrocellulose sheets (S&S) (13). Before blotting overnight, the gel was neutralized with 0.1 M Tris-HCl (pH 7.5) for 15 min and soaked together with the GeneScreen or nitrocellulose sheets in 10× SSC for another 15 min. After blotting, the filter was air-dried for 1 h at room temperature and baked for 4 h in an 80°C vacuum oven. The bands of ribosomal RNA could be seen clearly on the GeneScreen or nitrocellulose sheets under UV light after baking and their positions were accurately marked on the filter. For prehybridization, the filters were incubated with 5× Denhardt’s, 1 M NaCl at 65°C for 2 to 4 h.

Nick translated DNA probes (pZinc 37-11, pZinc 427) of 10⁶ cpm/μg specific activity were heated in a boiling water bath to complete denaturation (5 min) and added to the hybridization buffer (1 M NaCl, 1× Denhardt’s, 0.1% [w/v] SDS, 100 μg/ml calf thymus DNA, 5 mM NaPPi, 50 mM Tris-HCl [pH 7.5], 5

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**Fig. 1.** Bundle sheath cells (B) and MP (M) preparations from etiolated, greening, and green maize leaves, ×400. Light micrographs of preparations of BSS and MP, respectively, from etiolated maize leaves (a) and (b), 24 h greening leaves (c) and (d), and green leaves (e) and (f).

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added to dilute the lysate before application to the gel for analysis or storage at -70°C. A 10% (w/v) SDS polyacrylamide gel was run routinely to check the IVT products (3). Samples were prepared by mixing 10 μl of diluted lysate with 10 μl of 2× protein loading buffer (10 mM Tris-HCl [pH 8.6], 4% [w/v] SDS, 12% [w/v] sucrose, 5% β-mercaptoethanol). The gel was fixed with 10% (w/v) TCA, 10% (v/v) acetic acid and 30% (v/v) methanol before Enlightening (NEN) was applied for 15 to 30 min. The gel was dried and exposed to x-ray film (Kodak) at -70°C.

RESULTS

The Purity of BSS and MP Preparations. We have isolated MP and BSS from etiolated and greening leaves as well as from green leaves by combining mechanical and enzymic methods (Fig. 1, a–f). Although the plastids are not yet mature, the morphology, differential cell wall toughness, and physical arrangement of BSC and MC are fundamentally the same in etiolated and greening leaves as in green fully developed leaves. We have used only those preparations in which cross contamination was estimated to be less than 5% as judged by examination with the light microscope and comparisons of patterns of soluble and membrane proteins after SDS-PAGE (data not shown). Bundle sheath cells contaminating MC preparations usually appear as brick-shaped structures due to the cell wall remaining after digestion (7); for further analyses we used only MP preparations which had less than one brick-shaped cell per 100 MP. On the other hand, contaminating MC in preparations of BSS can be easily distinguished by their physical location; they are found between the BSS. Furthermore, the purity of the two cell type preparations was also evident from the distinctive IVT protein patterns resolved by SDS-PAGE as shown in Figure 2. In Vitro Translation products of BSS and MP RNAs. Samples of total RNA and poly A⁺ mRNA from BSS, MP, and whole leaves were translated in a RRL IVT system and the [35S]methionine labeled products were analyzed by SDS-PAGE as shown in Figure 2. A number of polypeptides (marked by arrows) were either BSC or MC specific. The most obvious ones were in the bands at about 100 kD (PEPCase and PPDK) in the MC RNA directed reactions, and at 55 and 18 kD for (RuBPCase LS and SS) in the BSS RNA directed translations. These products were identified by immunoprecipitation with specific antibodies (data not shown). Additional unidentified bands that are distinguishably different in the two cell types can be seen in the 60 to 80 kD regions and 25 to 55 kD regions.

Effect of Digestion Time on the Quality and Quantity of mRNA. We first prepared RNA from BSS and MP isolated from the same leaf material simultaneously by enzyme digestion but we were not satisfied with the quality of the RNA. We switched to a combination of enzymic and mechanical methods. However, we were concerned that the longer time required for the isolation of MP enzymically might affect the results of comparisons of the abundance of specific mRNAs in the two cell types at defined time points during greening. As shown in Figure 3, both the quality and quantity of mRNAs for RuBPCase LS and the 32 kD Qa protein of PSII did not change noticeably over the first 6 h of digestion of the leaf tissue even during the period of most rapid change (12–24 h) in mRNA accumulation (11). As noted in "Materials and Methods," MP were prepared by digestion for only 3 to 5 h.

Gene Expression: RuBPCase LS and β-ε CF, in BSC and MC during Greening. The effect of illumination on the levels of transcripts of the RuBPCase LS and β-ε CF genes during greening of BSC and MC is shown in Figure 4. In BSC, the 1.8 kb transcripts of the RuBPCase LS gene (4, 11) increased approximately 10-fold after 24 h of illumination and declined, while the 1.6 kb transcripts (4, 11) increased only a maximum of about 2-
fold. Neither the 1.6 kb nor 1.8 kb transcripts increased in MC; instead, the level of both transcripts dropped in the course of greening and eventually became undetectable. On the other hand, the level of transcripts complementary to the beta, epsilon CF₁ genes rose rapidly, reached a peak after 24 h of greening, and declined in both BSC and MC.

The ratio of transcripts encoded by these two adjacent plastid genes was dramatically different in BSC and MC during greening (examined by densitometer scanning). In BSC, the ratio (LS/β-ε CF₁) was roughly 10:1 in the dark and increased to 20:1 after 24 h of greening. It then declined to the original ratio after 96 h. However, in MC the ratio changed from 10:1 to 1:1 after 24 h of greening and further declined to less than 0.2:1 after 64 h of greening. Furthermore, in etiolated leaves both LS and SS RuBPCase were detected in the cell types by Western blotting; in green leaves, however, both polypeptides are present exclusively in BSC (J-Y Sheen, L Bogorad, unpublished data).

DISCUSSION

Transcripts of the RuBPCase LS gene are found in BSC but not MC of green leaves of maize. In contrast, the fused genes for the β-ε CF₁ that are adjacent to the LS gene on the maize chloroplast chromosome are expressed in both cell types (8, 10). Since the pools of transcripts of all of these genes increase upon illumination of dark-grown seedlings (4, 11, 12), we have investigated the patterns of pool-size changes in the two cell types during plastid development.

Our data show that about one-third of the RuBPCase LS mRNA in etiolated leaves is present in MC plastids. However, upon illumination, the level of transcripts declines in MC to become undetectable beyond 72 h of greening. The level of 1.8 kb transcripts of the LS gene increased approximately 10-fold after 24 h of illumination of etiolated leaves and the level of 1.6 kb transcripts increased approximately 2-fold over the same period and then dropped. By contrast, transcripts of the overlapping genes for the β-ε CF₁ increased about 10-fold after about 24 h of greening and then declined to a steady state level in both cell types. Although we have not demonstrated alterations in transcription rates per se directly and we have no information on the lifetimes of plastid mRNAs, the events that we have described suggest that transcription of the LS and β-ε CF₁ genes increased in BSC during the first 24 h of illumination of etiolated leaves and that the same pattern was followed for the β-ε CF₁ genes in MC, but in these latter cells, transcription of the large subunit genes is arrested, probably sometime during the initial phase of illumination. In essence, the RuBPCase LS gene is a photogene in BSC plastids but is a darkness-specific gene in MC plastids. It is possible, but seems unlikely, that the effects we have observed are solely the result of changes in RuBPCase LS mRNA-specific RNases.
The suppression of RuBPCase LS gene expression in MC of mature chloroplasts may be explained by the synthesis of a light-inducible repressor that blocks transcription, or by the light-mediated elimination of a transcription stimulator, or by a combination of both. Whatever the underlying mechanism, the transcription of CF1 genes is regulated differently. Furthermore, the morphological differentiation of BSC and MC is independent of events regulating RuBPCase LS expression.

Several lines of evidence argue that LS mRNA found in MC etioplast preparations in some early stages of greening were not due to contamination by BSC. First, the expression patterns of the gene are completely different during greening of the two cell types. Assuming cross-contamination is the same in preparations from each time point, if transcription of the LS gene is completely blocked in MC at all developmental stages, the overall expression patterns should be identical in the two cell types, although at a much lower level in MC. Second, at earlier developmental stages, LS transcripts in MC were found to be about 30% of those in BSC, while the level of cross-contamination is estimated to be less than 5%. Finally, sharp changes in the level of the 1.8 kb transcript seen in BSC are not apparent in RNA prepared from MP.

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LITERATURE CITED

1. AIVIN H, P LEDER 1972 Purification of biologically active globin mRNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad USA 69: 1408–1501
2. CHIRGWIN JM, AE PRZYBYLA, RJ MACDONALD, WJ RUTTER. 1979 Isolation of biologically active ribonuclease acid from source enriched in RNA ribonuclease. Biochemistry 18: 5294–5299
4. CROSSLAND LD, SR RODERMEL, L BOGORAD 1984 The single gene for the large subunit of RuBPCase in maize yields two differentially regulated mRNA. Proc Natl Acad Sci USA 81: 4060–4064
11. POULSEN C 1984 Two mRNA species differing by 258 nucleotides at the 5' end are formed from the barley chloroplast rbcL gene. Carlsberg Res Com- mun 49: 89–104