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Gene Expression of Chloroplast Translation Elongation Factor Tu during Maize Chloroplast Biogenesis

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We have examined the expression of a maize nuclear *tuf* gene (*tuf*A) coding for the chloroplast translation elongation factor EF-Tu. Southern analysis revealed that the maize chloroplast EF-Tu was encoded by at least two distinct genes in the nuclear genome. In order to know the effect of light on the expression of the *tuf*A gene during maize chloroplast biogenesis, we have analyzed the steady-state level of the *tuf*A mRNAs by Northern analysis. The steady-state level of the *tuf*A mRNAs was similar in both continuous light- and dark-grown seedlings. The level of the *tuf*A mRNAs also maintained at relatively same level during light-induced greening of etiolated seedlings and all examined developmental stages. These results indicate that the gene expression of the maize chloroplast EF-Tu is rarely light-regulated at it's mRNA level during chloroplast biogenesis.

Keywords: chloroplast EF-Tu, *tuf*A, light, maize (*Zea mays* L.)

The translational elongation factor plays a central role in protein synthesis. This GTP requiring protein interacts with aminoacyl-tRNA and delivers it to the ribosomal A-site during the translation elongation step. This function is performed by EF-Tu in prokaryotes and eukaryotic organelles, and by EF-1α in eukaryotes (Miller and Weissbach, 1977). In most lower photosynthetic eukaryotes, including *Chlamydomonas reinhardtii* (Baldauf and Palmer, 1990) and *Euglena gracilis* (Montadon and Stutz, 1983), EF-Tu (*tuf*A) is encoded in the chloroplast genome. However, higher plant chloroplast EF-Tu, which shows higher sequence homology to bacterial elongation factor than to cytoplasmic elongation factor, is encoded by nuclear DNA (Baldauf and Palmer, 1990; Baldauf *et al.*, 1990; Bonny and Stutz, 1993). Chloroplast EF-Tu is an abundant soluble protein and has been purified from spinach (Tiboni *et al.*, 1978), *E. gracilis* (Tiboni and Ciferri, 1986), and *Nicotiana tabacum* (Murayama *et al.*, 1993). A single nuclear *tuf*A gene was identified in *Arabidopsis thaliana* (Baldauf and Palmer, 1990) and multiple copies of this gene seemed to exist in the nuclear genome of other *Brassicaceae* (Baldauf *et al.*, 1990). In addition, EF-Tu is encoded by two genes in the tobacco (*N. sylvestris*) nuclear DNA (Murayama *et al.*, 1993) and by four genes in the soybean (*Glycine max*) (Bonny and Stutz, 1993).

Light is essential for normal chloroplast development in certain strains of algae and many higher plant species. The etioplasts of dark-grown plants lack chlorophyll and many chloroplast proteins. The illumination of etiolated cells causes chloroplast maturation and the expression of a number of chloroplast- and nuclear-encoded genes. The expression of many chloroplast-encoded genes appears to be regulated at the level of translation (Berry *et al.*, 1986; Klein and Mullet, 1987; Berry *et al.*, 1988; Klein *et al.*, 1988). The distribution of transcripts of the photosystem I chlorophyll a-apoproteins (protein products of *psa*A and *psa*B) in isolated barley plastids showed that these proteins were arrested on membrane-bound polysomes at the level of polypeptide chain elongation (Klein *et al.*, 1988). The translation of the large subunit protein (*rbc*L) of the ribulose-1,5-bisphosphate carboxylase has also been shown to be regulated in such a way in amaranth (Berry *et al.*, 1986). These results indicate that there are specific mechanisms for light regulation in the translation elongation step of individual chloroplast mRNAs.

The chloroplast protein synthesizing apparatus is essential for the development of the intact, functional

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organelles. Its capacity is regulated in a light-dependent fashion. In *E. gracilis*, where chloroplast protein synthesizing capacity is stimulated more than 10-fold by growth in the light (Reger *et al.*, 1972; Miller, 1983; Bouet *et al.*, 1986; Schwartzbach, 1990). The activities of several chloroplast protein synthesis factors (IF-2, IF-3, EF-Tu, EF-Ts, and EF-G) were light-induced (Breitenberger *et al.*, 1979; Fox *et al.*, 1980; Gold and Spremulli, 1985; Sreedharan *et al.*, 1985; Kraus and Spremulli, 1986). In higher plants, including soybeans and peas, their transcripts of the nuclear-encoded chloroplast elongation factors (EF-Tu and EF-G) were reported to accumulate in light-grown seedlings (Bonny and Stutz, 1993; Akkaya *et al.*, 1994). In contrast, these messages were not detected in dark-grown seedlings. When dark-grown soybean seedlings were illuminated, the steady-state level of the *tuf*A mRNA increased sharply within 3 hours (Bonny and Stutz, 1993).

Taken together, we may propose that the expression of the *tuf* gene could be important in controlling light-regulated chloroplast translation and thereby chloroplast development. The monocot leaf such as a maize leaf is an excellent model system for the study of chloroplast development, because it shows the gradient of chloroplast development from the leaf base to the leaf tip.

In this study, we have examined if the expression of the maize *tuf*A gene is light-regulated at the steady-state mRNA level as in the soybean *tuf*A gene during chloroplast biogenesis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Maize (*Zea mays* L. cv Golden X Bantam 70) seeds were purchased from Sakada Seeds Foundation (Japan). The seeds were germinated and grown in a growth chamber at 28°C under the 16 hour-light/8 hour-dark, 24 hour-light (continuous light), 24 hour-dark (continuous dark), or light-induction condition. During the light periods, plants were illuminated with a light intensity of 12,000 lux. For the light-induction condition, plants were grown in continuous dark for 7 days, and then illuminated for up to 48 hours. Leaves were harvested at the appropriate time points. Leaves of the dark-grown plants were harvested in complete darkness. Harvested leaves were immediately frozen in liquid nitrogen and kept at -70°C until needed.

Hybridization Probes

A 0.9 kb *Hae*III fragment of the tobacco *tuf*A gene (Sugita *et al.*, 1994) was used to detect tobacco and maize *tuf*A transcripts. A 0.58 kb *Pst*I fragment of the maize *rbc*L gene (Lee and Sim, 1995) was used to detect tobacco and maize *rbc*L transcripts. A 0.99 kb *Bam*HI-*Xho*I fragment of the radish 25S rDNA (Delseny *et al.*, 1983) was used to detect the tobacco and maize 25S rRNAs. The DNA fragments used as hybridization probes were excised from plasmid clones containing them, gel-purified, and radiolabeled using random priming kits (Boehringer Mannheim, Germany).

Preparation of DNA and Genomic Southern Blot Analysis

Genomic DNA was isolated from maize leaves according to the Dellaporta *et al.* (1983) protocol. Genomic DNAs were digested, separated on 0.7% agarose gel, and transferred to nylon membrane filters (Amersham) by capillary action (Sambrook *et al.*, 1989). Prehybridization was performed for 2-6 hours at 60°C in the presence of 5×SSC, 5×Denhardt's solution, 0.1% SDS, and 100 μg/ml of denatured salmon sperm DNA. Probe was added directly to the prehybridization solution and hybridization carried out for 18-24 hours at 60°C. Filters were washed once at room temperature and once at 60°C in 2×SSC, 0.1% SDS. For the detection of signal, autoradiography was done at -70°C with AGFA film in an intensifying screen.

Preparation of Leaf Tissues for the Electron Microscopy

Representative pieces of leaves were taken from seedlings at the two leaf stage. Samples were fixed for 2 hours under vacuum in 5% EM grade glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0). Samples were post-fixed in the cacodylate buffered 2% OsO_4. Tissues were washed three times with distilled water. Samples were stained *en bloc* with 2% aqueous uranyl acetate for 1 hour. Tissues were dehydrated through a series of graded ethanol (15%, 30%, 50%, 70%, 90%, and 100%). Samples were infiltrated with 1:1 EtOH/Spurr's resin for 1 hour, placed into 100% resin overnight, and then embedded and cured at 70°C overnight. Sections were cut on a ultramicrotome, picked up on 200 mesh copper grids, and stained with uranyl acetate and lead citrate. Sections were viewed and photographed on a JEM 100 CX-II electron microscope at 100 kV.

Preparation of RNA and Northern Blot Analysis

Total cellular RNA was prepared from primary leaves or secondary leaves by a guanidium thiocyanate extraction procedure (Nelson, 1994). 30 μg RNA per sample were fractionated by electrophoresis on 1.2% agarose/2.2 M formaldehyde gels. The RNA was transferred to Hybond N membranes (Amersham) using capillary method (Sambrook *et al.*, 1989), and then the blots were fixed by UV irradiation. The membranes were hybridized with probes at 37°C. The hybridization solution contained 50% formamide, 5× SSC, 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml of denatured salmon sperm DNA. The membranes were washed four times at 37°C for 10 min each in 2× SSC, 0.1% SDS and twice at 40°C for 10 min each in 1× SSC, 0.1% SDS. For the detection of transcripts, the membranes were scanned and then signals were quantitated by Phosphoimager (Fuji BAS).

RESULTS

A genomic Southern analysis was performed on maize genomic DNA in order to examine how many copies of the *tuf* genes exist in the maize genome. As shown in Fig. 1, two dense bands were detected in each maize genomic DNA that was digested with *Eco*RI, *Hind*III, *Bam*HI, or *Kpn*I, although several minor bands were also identified in genomic DNA lanes treated with *Eco*RI and *Hind*III. This suggests that at least two copies of EF-Tu genes are present in the nuclear genome of maize.

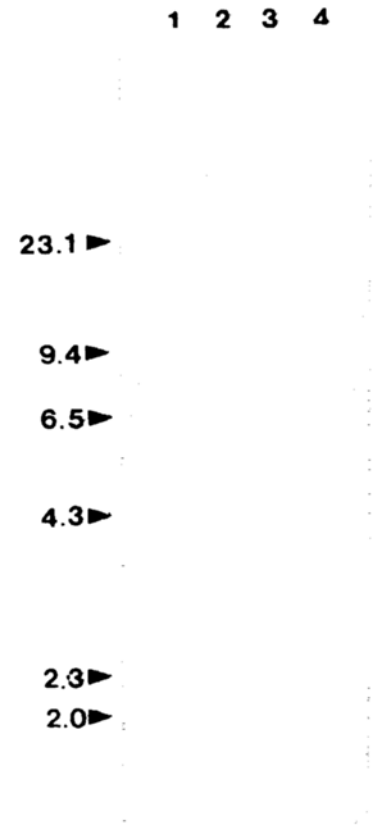

Fig. 1. Genomic Southern blot analysis. Maize genomic DNA was digested with *Eco*RI (1), *Hind*III (2), *Bam*HI (3), or *Kpn*I (4). The probe was the labelled 0.9 kb *Hae*III fragment of tobacco *tuf*A gene. The size markers (kb) shown on the left are λ DNA/*Hind*III digests.

To examine if the steady state *tuf* mRNA level is light-regulated, the maize seedlings were grown in the presence or absence of light and the dark-grown seedlings were illuminated for up to 48 hours. The ultrastructures of the plastids from the leaves of the maize seedlings grown under three different conditions were examined (Fig. 2). As expected, the light-grown maize leaf contained typical chloroplast and well developed thylakoid membrane stackings (Fig. 2a). The plastids from the leaves of the dark-grown seedlings contained the characteristic prolamellar bodies of etioplasts or less developed internal membranes (Fig. 2b). Fig. 2c shows that the etioplasts converted into chloroplasts slightly once the dark-grown seedlings were exposed to light.

Northern analysis was performed to detect the maize *tuf* transcript and measure its steady state levels under the three different conditions (Fig. 3). As a control, total RNA was also extracted from leaves of *Nicotiana tabacom* NC-82 grown under the 16 hour-light/8 hour-dark condition, because tobacco *tuf*A fragment was used as the probe to detect maize *tuf* transcripts. A single band, which size is similar to that of the tobacco *tuf*A transcript, was detected on the maize RNA lanes. As expected, the steady-state levels of the 25S rRNA transcripts were not changed under the conditions tested, indicating that this transcript is not regulated by light. However, the *rbc*L transcripts were significantly affected by light. The multiple *rbc*L mRNAs of similar sizes have been reported for several plant species (Crossland *et al.*, 1984; Rodermel and Bogorad, 1985). At the same conditions, the levels of the *tuf* transcripts did not change significantly, indicating that the expression of the maize *tuf* gene is not light-regulated significantly at the steady state mRNA level. This is in contrast to the expression of the soybean *tuf* gene which is light-regulated at the mRNA level (Bonny and Stutz, 1993).

The effect of light upon the level of the maize *tuf* transcript during shorter greening periods was also

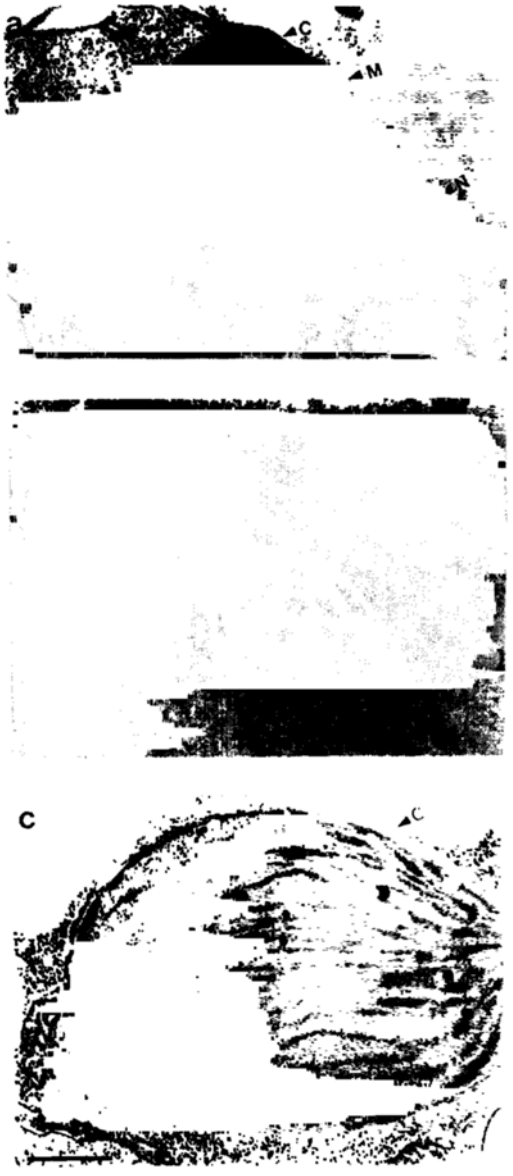

Fig. 2. Electron micrographs of plastids from the middle of a fully expanded first leaf of maize seedlings. a. plastids from 7-day-old first leaf grown in light (16 hour-light/8 hour-dark), b. plastids from 7-day-old first leaf grown in darkness, c. plastids from 7-day-old first leaf grown in transition of darkness to light. Arrows show chloroplast (C), mitochondria (M), and nucleus (N), respectively. Bars=2 μm.

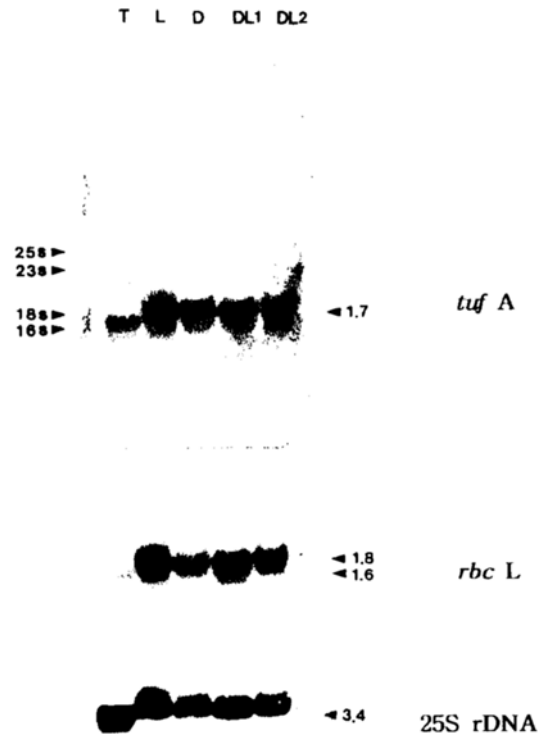

Fig. 3. Northern blot analysis. Total RNAs were isolated from leaves grown in continuous light (9 days, L), in continuous darkness (9 days, D), and in transition of continuous darkness (8 days) to light for 24 hours (DL1) and 48 hours (DL2). 30 μg of total RNA were loaded on each lane and hybridized with random-primed probes from *tuf*A, *rbc*L, or 25S rDNA. Tobacco total RNA (20 μg) was also loaded as a control (T). Size markers are maize cytoplasmic rRNAs (25S and 18S) and chloroplast rRNAs (23S and 16S). The sizes of the *tuf*A, *rbc*L, and 25S rDNA transcripts are indicated in kilobases. The tufA-blotted membrane was deprobed in 0.1×SSC. 0.1% SDS for 3 hours, then reprobed with *rbc*L or 25S rDNA, respectively.

examined, because *tuf*A mRNA accumulation was an early event during the greening process in *Chlamydomonas* and soybean (Bonny and Stutz, 1993; Silk and Wu, 1993) and this may be also true in maize. Fig. 4 shows the patterns of each transcript upon the less-than-24 hour illumination of etiolated plants. The accumulation of *tuf* mRNA was not observed up to the 6 hour illumination of dark-grown seedlings, but slightly increased after the 24 hour illumination. Changes in the levels of *rbc*L transcripts were also measured under the same condition and showed the similar pattern that was already known (Crossland *et al.*, 1984; Rodermel and Bogorad, 1985). As reported previously, the light-regulation pattern between 1.8 kb and 1.6 kb of *rbc*L transcripts was different from each other (Crossland *et al.*, 1984; Rodermel and Bogorad, 1985). These results suggest that the *tuf* transcript of a maize is hardly accumulated in early process of greening, either.

It was also examined if the steady state level of the maize *tuf* mRNA was regulated at developmental stage. To this purpose, maize seedlings were grown from 7 to 9 days under conditions identical to those

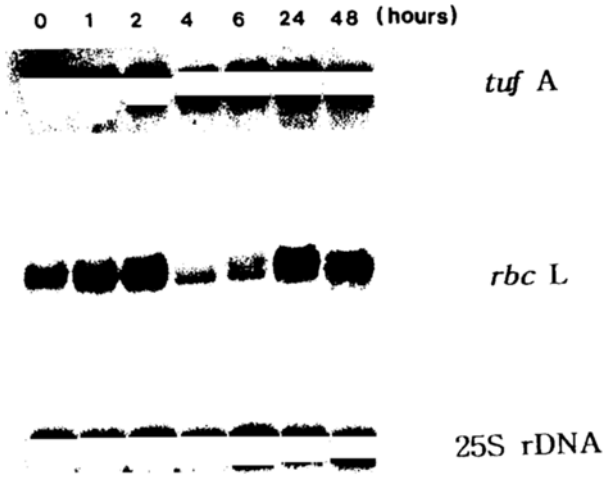

Fig. 4. The changes of maize leaf mRNA during greening. Dark-grown seedlings (7 days) were illuminated and total RNA was isolated at the indicated hours (0, 1, 2, 4, 6, 24, and 48 hours). 30 μg of total RNA were loaded on each lane and hybridized with random-primed probes from *tuf*A, *rbc*L, or 25S rDNA. Northern blots with the *tuf*A, *rbc*L, or 25S rDNA probe were performed under the same conditions described in Fig. 3.

described above. Fig. 5a shows the accumulation of maize leaf mRNAs during light- and dark-growth. The kinetics of accumulation at various conditions of each RNA transcript were shown in Fig. 5b. The *tuf* transcript was detected at all the developmental stages and maintained at relatively same level. However, the level of *rbc*L transcript was considerably lower in dark-grown plants than in light-grown controls and increase rapidly upon illumination. As shown in Fig. 5b, the nucleus-encoded *tuf* gene was expressed at early developmental phases preceding the expression of chloroplast-encoded *rbc*L gene. It has been reported that the expression of nucleus-encoded ribosomal proteins preceded the plastid gene expression in spinach (Harrak *et al.*, 1995). Although the expression level of each gene was analyzed at narrow scale, the results suggested the *tuf* transcript was not changed to significant extent during developmental stage.

DISCUSSION

The *tuf*A gene, encoding chloroplast translational elongation factor Tu, was initially identified as a ch-

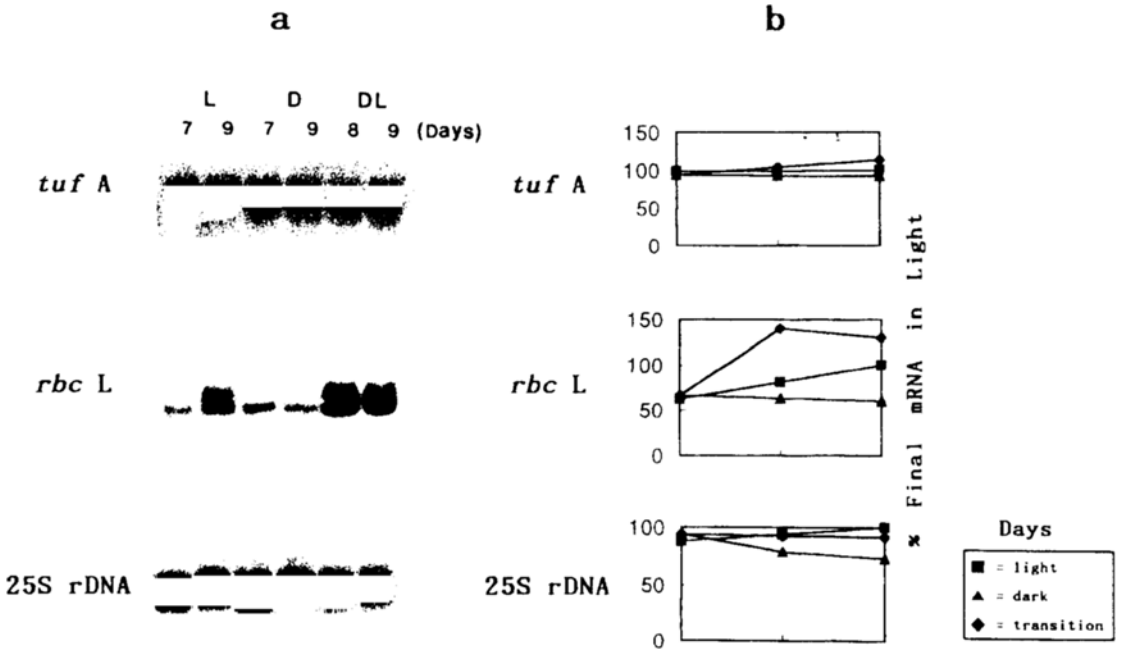

Fig. 5. Accumulation of maize leaf transcripts during light growth and dark growth. (a) Northern analysis of maize leaf transcript. Total RNA was prepared from leaves grown in continuous light (L), or continuous darkness (D) for the indicated days. At 7 days, dark-grown plants (D-L) were illuminated and total RNA was isolated. 30 μg of total RNA were loaded on each lane and hybridized with random-primed probes from *tuf*A, *rbc*L, or 25S rDNA. Northern blots with the *tuf*A, *rbc*L, or 25S rDNA probe were performed under the same conditions described in Fig. 3. (b) The relative amounts of the *tuf*A, *rbc*L, and 25S rDNA transcripts to the levels found in leaves grown for 7 to 9 days in continuous light were measured. Filled rectangle (■), filled triangle (▲), and filled diamond (◆) designate mRNA levels in light-grown, dark-grown, and dark-to-light transferred seedlings, respectively.

loroplast gene in the green algae, *C. reinhardtii* by genomic Southern analysis (Watson and Surzycki, 1982). However, the *tuf* homologues were mapped on the nuclear genomes of other higher plants. Multiple nuclear genes encoding chloroplast EF-Tu have been found in the cruciferous Brassica species (Baldauf *et al.*, 1990) and a single nuclear *tuf*A gene was mapped in *A. thaliana* (Baldauf and Palmer, 1990). Tobacco (*N. sylvestris*) and soybean also contain two *tuf* genes and four *tuf* homologues in their nuclear genomes, respectively (Bonny and Stutz, 1993; Murayama *et al.*, 1993). In this study, we have identified the copy number of chloroplast translational elongation factor Tu in a maize. Southern hybridization data suggests that the maize nuclear genome contains at least two distinctive *tuf* genes (Fig. 1). However, given several minor bands in *Eco*RI and *Hind*III-digested maize DNAs, there is a possibility that the *tuf* gene exists as a multigene family in a maize.

Chloroplast protein synthetic apparatus plays a role in coordinating nuclear events with chloroplast gene expression during light-induced chloroplast differentiation (Taylor, 1989). In barley, chloroplast-encoded transcription and translation apparatus genes were transcribed before chloroplast photosynthetic genes were expressed during chloroplast development (Baungautner *et al.*, 1993). Transcription of nucleus-encoded plastid ribosomal protein genes also preceded that of plastid-encoded genes during early phases of chloroplast development in spinach (Harrak *et al.*, 1995). In soybean and pea, the steady state mRNA levels of the nucleus-encoded chloroplast elongation factors (EF-Tu and EF-G) were reported to be light-regulated (Bonny and Stutz, 1993; Akkaya *et al.*, 1994). However, our results indicate that the *tuf* mRNA level is not regulated to significant extent by illumination in maize. The effect of light on the steady state mRNA level of other plastid proteins has also been shown to vary among different plant species. For example, *rbc*L and/or *rbc*S mRNA levels have been shown to be strongly light-regulated in peas (Smith and Ellis, 1981) and soybeans (Berry-Lowe *et al.*, 1982), moderately in maizes (Nelson *et al.*, 1984) and amaranths (Berry *et al.*, 1986), but rarely in barleys (Klein and Mullet, 1987). Light effect on plastid proteins level has also been known to vary among different plant species (Mayfield *et al.*, 1995). Research on these aspects of the *tuf* gene expression is currently in progress.

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