Plant Physiol. (1987)84, 808–813
0032-0889/87/84/0808/06/$01.00/0

Effect of Tagetitoxin on the Levels of Ribulose 1,5-Bisphosphate Carboxylase, Ribosomes, and RNA in Plastids of Wheat Leaves

Received for publication October 27, 1986 and in revised form March 6, 1987

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ABSTRACT

Growth of wheat seedlings in the presence of the phytotoxin tagetitoxin produces pigment-deficient leaves of normal size and morphology whose cells contain only rudimentary plastids. We could not detect the accumulation of either the plastid-encoded large subunit or the nuclear-encoded small subunit of the chloroplast stromal enzyme ribulose 1,5-bisphosphate carboxylase (RuBPCase) in western blots of protein extracted from leaves of such seedlings. Sucrose gradient centrifugation profiles showed that plastid ribosomes were essentially absent in toxin-treated leaf tissue while cytoplasmic ribosomes were relatively unaffected. Northern blot analysis of RNA in toxin-treated leaves showed a deficiency of plastid ribosomal RNA (16S and 23S) as well as reduced levels of plastid mRNAs for the large subunit of RuBPCase and for the 32 kilodalton thylakoid Q_B polypeptide. Northern analysis also showed that the nuclear-encoded rbcS mRNA for the small subunit of RuBPCase is present in only trace amounts in toxin-treated leaves.

Tagetitoxin is a bacterial phytotoxin, produced by *Pseudomonas syringae* pv *tagetis*, which causes chlorosis in developing but not mature leaves (22, 27). Although the mode of action of tagetitoxin is not known, ultrastructural and physiological evidence indicate that its effects are relatively plastid specific (13, 17). Treated wheat seedlings grow at a normal rate and develop first and second leaves of standard size and morphology, but these leaves are pigment deficient and contain rudimentary plastids. The chloroplasts in affected leaves have intact envelopes but severely disorganized and greatly reduced internal membrane systems. Etioplasts in dark-grown, toxin-treated leaves lack organized prolamellar bodies. The activity of the major stromal enzyme RuBPCase is essentially absent. Furthermore, according to the ultrastructural evidence, plastid ribosomes in both light- and dark-grown leaves appear to be depleted by toxin treatment.

In this report, we examine further the effects of tagetitoxin on various plastid components. We find that tagetitoxin largely prevents the accumulation of not only both the large and small subunits of RuBPCase themselves but of their respective plastid-encoded and nuclear encoded mRNAs as well. In addition, another major plastid mRNA for a thylakoid protein (32 kD Q_B) fails to accumulate. Furthermore, toxin treatment results in the selective depletion of plastid (70S) ribosomes and their component 16S and 23S rRNAs vis-à-vis their cytosolic (80S ribosome) counterparts.

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MATERIALS AND METHODS

Plant Material. Seedlings of spring wheat (*Triticum aestivum* L. cv Lathrop, University of Wisconsin seed lots, harvested 1978–1982) were grown on filter paper at 22°C in darkness or 12-h photoperiods (cool-white fluorescent bulbs, Sylvania, 281 ± 10 μE m^{-2} s^{-1}). Seedling root systems were moistened with either distilled water or 50 μM tagetitoxin. The seedlings were harvested between 6 and 10 d after germination, when the first leaves were 8 to 12 cm long.

Ribosome Isolation. Ribosomes were isolated essentially as described by Feierabend and Schrader-Reichhardt (6) from the first leaves of 50 to 70 9-d-old wheat seedlings which had been immersed for 2 h in H_2O bubbled with N_2 to dissociate polyribosomes into monomeric ribosomes (16). Dark-grown seedlings were handled under a green safe light until leaf tissue was homogenized. The upper 5 mm and lower 20 mm of each leaf were discarded, and the remaining leaf tissue for each treatment was homogenized with a mortar and pestle in 12 ml 0.2 M sucrose, 0.1 M Tris-HCl (pH 7.5), 10 mM KCl, 10 mM $MgCl_2$, and 4 mM DTT. Final ribosomal pellets were resuspended with gentle shaking for 4 h at 4°C in 0.5 ml 40 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 20 mM KCl. Between 2.5 and 4.8 A_{260} units of resuspended ribosomes in a total volume of 0.5 ml were loaded onto similarly buffered 10 to 34% linear sucrose gradients which were centrifuged for 4 h at 25,000 rpm (85,000g_{av}) in a Beckman SW 28 rotor. Gradients were scanned at 260 nm with an ISCO density gradient fractionator.

RNA Isolation. Total leaf RNA was isolated from 7- to 9-d-old seedlings according to modifications of the methods of Chirgwin *et al.* (5) and Glisin *et al.* (9). Approximately 100 leaves (2.5 g fresh weight) were powdered in liquid N_2, and subsequently homogenized in a polytron for 15 s with 12 ml buffer containing 4 M guanidinium thiocyanate, 50 mM Tris-HCl (pH 7.6), 10 mM EDTA, 2% (w/v) sodium *N*-lauroyl sarcosine, 1% (v/v) β-mercaptoethanol, and 10 mM vanadyl ribonucleoside complex (2). The homogenate was centrifuged at 27,000*g* for 10 min, and the supernatant brought to 2.4 M CsCl and centrifuged at 34,800*g*. The supernatant was layered over a 1 ml cushion containing 5.7 M CsCl in 0.1 M EDTA (pH 7.5). RNA was separated from DNA by pelleting through the CsCl cushion at 35,000 rpm (120,500*g*) for 18 h in a Beckman SW 60 rotor. The pellet was dissolved in a small amount of 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 1% (w/v) SDS (TES) and extracted with an equal volume of 4:1 chloroform: butanol. The organic phase was reextracted with an equal volume of TES, the aqueous phases were combined, and RNA was ethanol-precipitated and stored at −20°C.

Purification of RuBPCase. Approximately 40 to 50 leaves were harvested from 1- to 2-week-old wheat seedlings grown in vermiculite with deionized water at 22°C with a 12 h photoperiod.

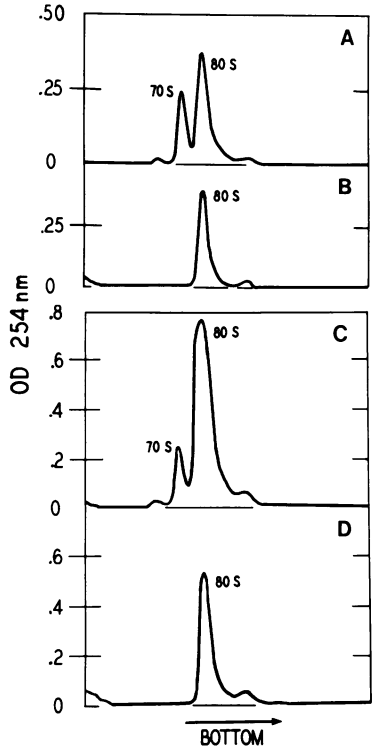

FIG. 1. Profiles of sucrose gradient separations of monoribosomes isolated from first leaves of 9-d-old wheat seedlings grown in H_2O or in 50 μM tagetitoxin, either in the light or dark. Total A_{260} units loaded per gradient were normalized within light and dark treatments. A, H_2O, light; B, tagetitoxin, light; C, H_2O, dark; D, tagetitoxin, dark.

Initial steps for the purification of RuBPCase[2] from harvested leaves were based on the protocol of Hall and Tolbert (11). Washed, sliced leaves were ground in a Waring blendor for 1 to 2 min in 150 to 200 ml grinding buffer consisting of 50 mM Tricine-NaOH (pH 8.0), 1 mM EDTA, 5 mM DTT, and 2% (w/v) insoluble PVP. The homogenate was filtered through five layers of cheesecloth and one layer of Miracloth and centrifuged at 23,000g for 45 min. The supernatant was decanted through one layer of Miracloth and brought to a final concentration of 18% PEG 4000. After 30 min of stirring, the solution was again centrifuged at 23,000g for 45 min, and the supernatant was decanted into a cold beaker. Protein (including RuBPCase) was precipitated from the supernatant by the addition of 2 M $MgCl_2$ with stirring to bring the final concentration to 20 mM $MgCl_2$. The precipitated protein was pelleted by centrifugation at 16,000g for 30 min, and the pellet was taken up in 10 to 12 ml grinding buffer. After a further centrifugation at 27,000g for 30 min, the clarified solution was layered onto a DEAE cellulose (Whatman DE52) column (2.5 × 20.5 cm) equilibrated with grinding buffer (minus PVP). The RuBPCase was eluted with a linear gradient of 0.0 to 0.32 M $NaHCO_3$ in grinding buffer, and was precipitated from the peak A_{280} fractions by the addition of 1.5 volumes of saturated ammonium sulfate. The RuBPCase was further purified by a variation of the sucrose gradient centrifugation technique described by Goldthwaite and Bogorad (10). The precipitated RuBPCase was resuspended in grinding buffer and layered onto 10 to 30% linear sucrose gradients made up in 25 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, 25 mM KCl, and 5 mM DTT. The gradients were centrifuged for 32 h at 25,000 rpm (85,000g_{av}) in a Beckman SW28 rotor and subsequently fractionated and scanned at 280 nm with an ISCO density gradient fractionator. Peak RuBPCase fractions were pooled and precipitated as for DEAE cellulose fractions.

The subunits of purified RuBPCase were separated electrophoretically on 1.5 mm 12% SDS-polyacrylamide slab gels according to Laemmli (14). Excised gel pieces were lyophilized for 24 h, ground in a mortar to a very fine powder, and used directly for the immunization of rabbits to produce antisera.

Preparation of Antisera. Antisera against the electrophoretically separated subunits of RuBPCase were prepared by immunization of New Zealand white rabbits with powdered polyacrylamide containing the SDS-denatured subunits, essentially as described by Franssen *et al.* (8). Powdered gels containing either approximately 1 mg large subunit or 0.4 mg small subunit were mixed with 1 to 2 ml phosphate buffered saline (10 mM sodium phosphate [pH 7.2], 0.9% NaCl) and emulsified with an equal volume of Freund's complete adjuvant (Difco). The emulsions were injected subcutaneously at multiple sites on the backs of the rabbits. Booster injections of powdered gels containing approximately 0.6 mg large or 0.3 mg small subunit emulsified with Freund's incomplete adjuvant were administered after 4 weeks. Beginning 1 week after the booster injection, 20 ml of blood were collected from an ear vein once a week for 2 weeks. The collected blood was allowed to clot for 1 h at room temperature, then for 10 to 16 h at 4°C. The clot was spun down at

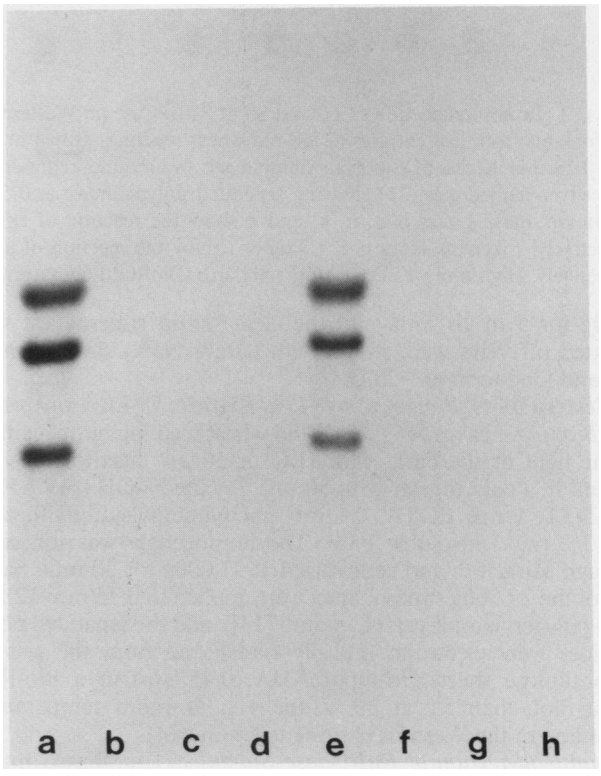

FIG. 2. Northern analysis of chloroplast rRNA abundance in leaves of 7- to 8-d-old wheat seedlings. Total RNA was hybridized with a nick-translated probe specific for 16S and 23S rRNA. RNA is from tissue grown in light with no toxin treatment (lane a), light with 50 μM tagetitoxin (lanes b, c, d), dark with no toxin treatment (lane e), dark with 50 μM tagetitoxin (lanes f, g, h). The amount of total RNA loaded in each lane was 5 μg (lanes a, b, e, f), 10 μg (lanes c, g), and 20 μg (lanes d, h).

[2] Abbreviations: RuBPCase, ribulose 1,5-bisphosphate carboxylase (LS = large subunit; SS = small subunit); kbp, kilobase pair; LHCP, light-harvesting Chl a/b protein; Q_B, 32 kD PSII herbicide-binding protein; rbcL, chloroplast gene for large subunit of RuBPCase; rbcS, nuclear gene for small subunit of RuBPCase; psbA, chloroplast gene for 32 kD Q_B polypeptide; SSC, sodium saline citrate.

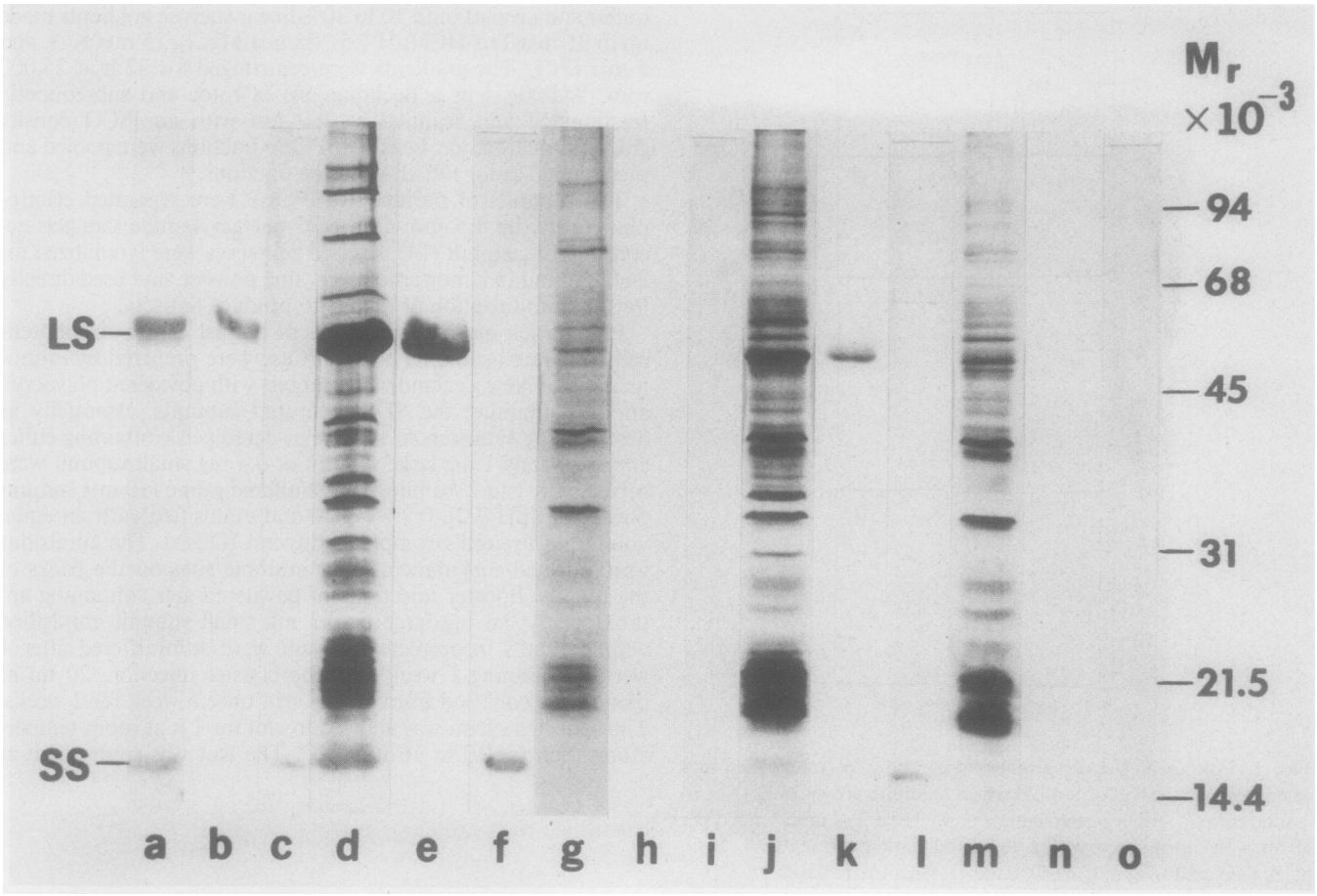

FIG. 3. Immunodetection of LS and SS of RuBPCase on Western blots of electrophoretically separated SDS-treated polypeptides in the 27,000*g* supernatants from leaf extracts of 9-d-old wheat seedlings grown in the light or dark either in H_2O or in 50 μM tagetitoxin. Samples were loaded onto 0.75 mm 12.5% SDS-polyacrylamide gels in amounts representing approximately equal leaf fresh weights. Lanes a, d, g, j, and m are strips from silver-stained gels (21) showing separated polypeptides: a, 0.5 μg purified RuBPCase; d, H_2O, light; g, tagetitoxin, light; j, H_2O, dark; m, tagetitoxin, dark. Lanes b, e, h, k, and n show the reaction of anti-LS serum (1:3000 dilution) with Western blots of lanes a, d, g, j, and m, respectively. Likewise, lanes c, f, i, l, and o show the reaction of anti-SS serum (1:3000 dilution) with Western blots of lanes a, d, g, j, and m, respectively. High mol wt SDS-PAGE standards (BioRad Laboratories) were used for mol wt determinations.

5000*g* for 5 to 10 min, and the clear serum supernatant was pipetted off. Sera were stored with 0.02% NaN_3 short-term at 4°C and long-term at −20°C.

Western Blots. *Preparation of Leaf Extracts for Electrophoresis and Blotting.* Leaves of 7- to 9-d-old wheat seedlings grown either in the light or the dark, with H_2O or 50 μM tagetitoxin, were ground in a cold mortar with 50 mM Tricine-NaOH (pH 7.5), 5 mM DTT, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluride, and 2% (w/v) insoluble PVP. The homogenate was squeezed through Miracloth and centrifuged at 27,000*g* for 30 min. Samples of the 27,000*g* supernatants were applied to 0.75 mm 12.5% SDS-polyacrylamide gels (Laemmli [14]), and the separated polypeptides were electrophoretically transferred from the gels to nitrocellulose sheets (Millipore, HA, 0.45 μm) in a Bio-Rad Trans-Blot chamber at 60 V for 4 h at room temperature according to the Western blotting technique (4).

Immunodetection of RuBPCase Subunits. The blotted nitrocellulose sheets were taken through a series of washes and incubations with primary and secondary (indicator) antibodies according to the method of Titus *et al.* (26). Primary rabbit antibodies directed against LS and SS were used at dilutions of 1:200 to 1:4000. Blots were secondarily incubated with alkaline phosphatase-linked goat anti-rabbit antibody (Kirkegaard & Perry Laboratories, Inc., 0.1 mg/ml) diluted 1:1000. Bound antibodies were detected with 5-bromo-4-chloro-3-indolyl phosphate (Sigma No. B8503) as substrate in an agarose gel matrix.

Northern Blots. Total leaf RNA was denatured by glyoxylation (20) and electrophoresed on horizontal 1.2% agarose gels with Tris-Borate-EDTA (pH 8.3) as the running buffer. RNA was blotted onto Gene Screen filters (New England Nuclear) and fixed by baking the filters at 80°C for 2 to 4 h. Filters were presoaked for at least 4 h at 42°C in 50% formamide, 3 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na-citrate), 1 × Denhardt's solution, 0.1 mg/ml denatured calf thymus DNA, and 0.2% SDS, and were subsequently hybridized in the same buffer containing between 10^6 and 10^7 cpm of denatured, nick-translated, chloroplast DNA probe for 24 h at 42°C. Filters were washed for 1.5 h at 65°C in 2 × SSC, 0.5% SDS and 1.5 h at 65°C in 1 × SSC, 0.1% SDS, then air-dried and exposed to Kodak XAR-5 film (Eastman Kodak) at −80°C with an intensifying screen (DuPont de Nemours Co.).

Chloroplast DNA Probes. Chloroplast gene probes constructed in pBR322 were the gift of L. Bogorad (Harvard University, Cambridge, MA). Plasmid pZmc461 contains a 580 bp subfragment of maize BamHI fragment 9 internal to the rbcL gene; pZmc427 contains a 1.9 kbp subfragment of maize BamHI fragment 8 which includes the entire psbA gene for the Q_B thylakoid polypeptide; pZmc100 contains the 6 kbp maize EcoRI fragment a (15) which lies within the inverted repeat and includes the 16S and 23S rRNA genes. A probe specific for an entire

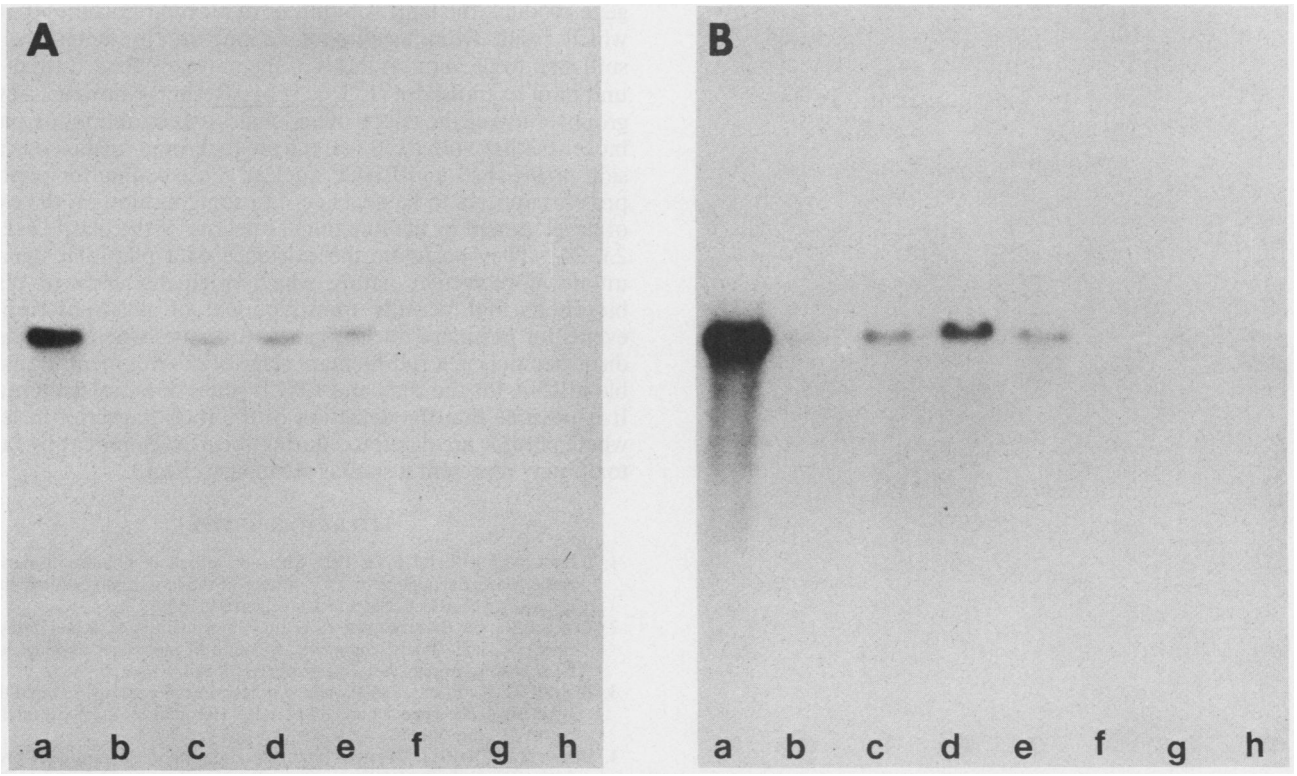

FIG. 4. Northern analysis of chloroplast mRNA abundance in leaves of 7- to 8-d-old wheat seedlings. Total RNA was hybridized with nick-translated chloroplast gene probes specific for the rbcL transcript (A) or psbA transcript (B). RNA is from tissue grown in light with no toxin treatment (lane a), light with 50 μM tagetitoxin (lanes b, c, d), dark with no toxin treatment (lane e), dark with 50 μM tagetitoxin (lanes f, g, h). The amount of total RNA loaded in each lane was 5 μg (lanes a, b, e, f), 10 μg (lanes c, g), and 20 μg (lanes d, h).

nuclear-encoded rbcS gene of wheat (pTaYSs3.2 which contains a 3.2 kbp BamHI genomic fragment) was the gift of Michael Murray (Agrigenetics Advanced Research Div., Madison, WI). Plasmid DNA was isolated by the alkaline lysis method and radioactively labeled by nick-translation essentially as described by Maniatis *et al.* (18).

RESULTS

Ribosomes and Ribosomal RNA. Total leaf RNA levels are typically depleted by about 50 and 25%, respectively, in light- and dark-grown toxin-treated seedlings. Plastid (70S) ribosomes are absent from the sucrose gradient fractionation profiles of ribosomal preparations made from leaves of toxin-treated seedlings grown either in the light (B) or the dark (D) (Fig. 1). Northern analysis using a probe specific for the chloroplast 16S and 23S rRNAs verifies that these rRNA species are not detectable in 7-d-old toxin-treated leaves (Fig. 2).

Cytoplasmic (80S) ribosomes are present in approximately equal abundance in both untreated and treated leaves (Fig. 1). As judged by the intensity of their ethidium bromide staining on gels, the two major RNA species of the cytoplasmic ribosomes (25S and 18S) are present in essentially normal amounts in total leaf RNA preparations (gel not shown).

Immunodetection of RuBPCase Subunits. Antisera produced against the subunits of RuBPCase reacted with their respective antigens on Western blots of SDS-treated polypeptides from untreated wheat leaf extracts, but failed to detect either subunit on blots of polypeptides from toxin-treated leaf extracts (Fig. 3). This was true for all immunodetection experiments, which were conducted over a wide range of antisera dilutions (1:200 to 1:4000) and of leaf extract loadings onto gels used for Western blotting. No large or small subunit polypeptides were detected in either 27,000*g* supernatants (soluble fraction) or pellets (membrane fraction) (not shown) of toxin-treated extracts.

While it is obvious from gels such as that shown in Figure 3 and longer gels of higher resolution (not shown) that a number of polypeptides are far less abundant than normal in the soluble fraction of toxin-treated extracts (particularly of light-grown leaves), there are only a few major bands other than the RuBPCase subunits that appear to be completely absent. These include a doublet at M_r 90,000, a singlet at M_r 65,000, a doublet at M_r 38,000 and a singlet at M_r 31,000. No prominent novel bands appear in toxin-treated leaf extracts.

Presence of Chloroplast-specific mRNAs. Wheat seedlings grown in tagetitoxin accumulate only trace amounts (estimate, 1–2% of normal) of two major plastid-encoded mRNAs, the rbcL transcript for the large subunit of the soluble RuBPCase and the psbA transcript for the thylakoid 32 kD Q_B polypeptide (Fig. 4). (Additional data, not shown here, indicate that the transcript for the β and ϵ subunits of the thylakoid membrane-bound CF_1 multimeric protein is similarly depleted.)

Furthermore, the nuclear-encoded rbcS transcript for the small subunit of RuBPCase is also depleted to approximately 1 to 2% of its normal level (Fig. 5).

DISCUSSION

The evidence presented here verifies that plastid ribosomes and their component rRNAs are selectively depleted in both light- and dark-grown, toxin-treated tissue. The lack of the 23S and 16S plastid rRNA species indicates that they are either not transcribed or, alternatively, that they are unstable in the absence of assembled ribosomes. Because Northern analyses assay only the steady-state levels of transcripts, and we have no measurements of the rates of synthesis or turnover of any of the RNA species, it is possible that increased turnover of RNA may account for at least some of their depletion in leaves of 7-d-old

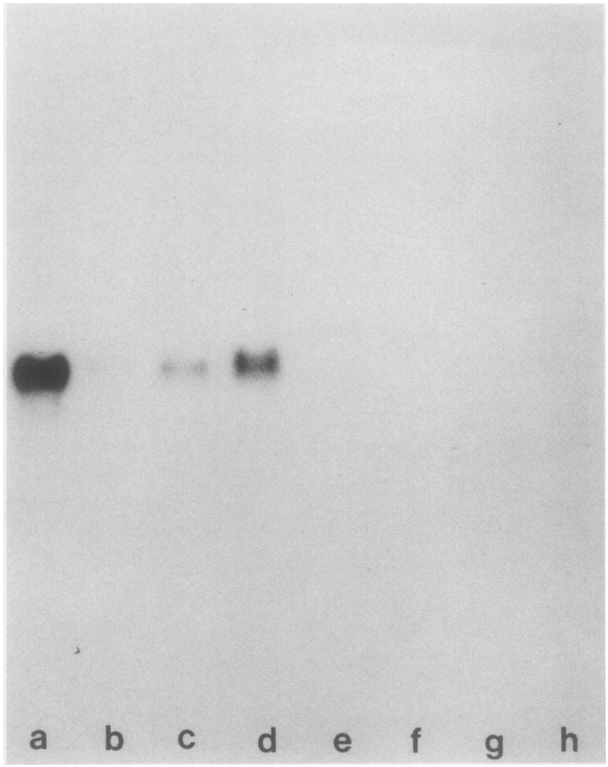

FIG. 5. Northern analysis of the abundance of the nuclear-encoded rbcS transcript for the SS of RuBPCase in leaves of 7- to 8-d-old wheat seedlings. Total RNA was hybridized with the nick-translated rbcS probe pTaYSs3.2. RNA is from tissue grown in light with no toxin treatment (lane a), light with 50 μM tagetitoxin (lanes b, c, d), dark with no toxin treatment (lane e), dark with 50 μM tagetitoxin (lanes f, g, h). The amount of total RNA loaded in each lane was 5 μg (lanes a, b, e, f), 10 μg (lanes c, g), and 20 μg (lanes d, h).

seedlings. The accompanying substantial depletion of the several chloroplast-encoded mRNA species assayed, however, makes it plausible to suggest that the transcription of chloroplast genes may be generally repressed (*i.e.* for all types of genes) in toxin-treated leaves. Experiments are in progress using isolated chloroplasts and chloroplast lysates to determine whether tagetitoxin can act directly as an inhibitor of plastid transcription *in organello* or *in vitro*.

In light of the depletion of both the rbcL transcript and plastid ribosomes, it is not surprising that the large subunit of RuBPCase fails to accumulate in toxin-treated leaves. Additionally, however, we find that the nuclear-encoded rbcS transcript is greatly depleted and its gene product, the small subunit, is undetectable in toxin-treated leaves. It would appear that tagetitoxin, whose physiological effects seem to be largely specific for early events in plastid development (17), also affects the accumulation of at least one nuclear-encoded transcript for a chloroplast-localized polypeptide. It should be noted that rbcS transcript depletion occurs in leaves that exhibit normal morphogenesis and anthocyanin accumulation and other physiological and ultrastructural characteristics (this paper and Ref. 17) which suggest that a significant number of nonplastidial nuclear genes, as well as the cytoplasmic translational apparatus, function normally. However, since we have no evidence concerning the abundance of other chloroplast-specific and nonchloroplast-specific nuclear transcripts in toxin-treated leaves, we are unable to draw any conclusions regarding the specificity or the significance of the effect of the toxin on the rbcS transcript.

Studies concerning the coordination of nuclear and plastid genome expression have established that the absence of the rbcL gene product, the large subunit, or of plastid ribosomes (both of which result from tagetitoxin action) are in themselves not sufficient to prevent synthesis of the nuclear-encoded small subunit or of its transcript (1, 3, 6, 7, 24). Recently, however, several groups studying the effects of herbicides and mutations on plastid biogenesis have observed that the phytochrome-mediated expression of the rbcS and LHCP nuclear genes coding for plastidial proteins appears to be dependent in some fashion on the extent of development or the functional integrity of the plastid (12, 19, 23, 25). They postulate the existence of a plastidial signal of unknown molecular nature which originates early in plastid biogenesis and permits transcriptional or posttranscriptional events for pertinent nuclear genes. In tissues where plastids are disturbed during a rudimentary stage of development, translatable mRNA for the rbcS and LHCP genes does not accumulate. It is possible that the depletion of the rbcS transcript in leaves where plastids are disrupted during their development by tagetitoxin may represent a similar secondary effect.

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