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Guanosine tetra- and pentaphosphate synthase activity in chloroplasts of a higher plant: association with 70S ribosomes and inhibition by tetracycline

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ABSTRACT

Chloroplasts possess bacterial-type systems for transcription and translation. On the basis of the identification of a *Chlamydomonas reinhardtii* gene encoding a RelA-SpoT homolog (RSH) that catalyzes the synthesis of guanosine tetra- or pentaphosphate [(p)ppGpp], we have previously suggested the operation of stringent control in the chloroplast genetic system. Although RSH genes have also been identified in several higher plants, the activities of the encoded enzymes and their mode of action in chloroplasts have remained uncharacterized. We have now characterized the intrinsic (p)ppGpp synthase activity of chloroplast extracts prepared from pea (*Pisum sativum*). Fractionation by ultracentrifugation suggested that the (p)ppGpp synthase activity of a translationally active chloroplast stromal extract was associated with 70S ribosomes. Furthermore, this enzymatic activity was inhibited by tetracycline, as was the peptide elongation activity of the extract. Structural comparisons between rRNA molecules of *Escherichia coli* and pea chloroplasts revealed the conservation of putative tetracycline-binding sites. These observations demonstrate the presence of a ribosome-associated (p)ppGpp synthase activity in the chloroplasts of a higher plant, further implicating (p)ppGpp in a genetic system of chloroplasts similar to that operative in bacteria.

INTRODUCTION

In bacteria, stringent control plays an important role in the adaptation of cell physiology to adverse environmental conditions such as nutrient limitation. When amino acids are in short supply, the 70S ribosome senses the associated increase in the proportion of deacylated tRNA molecules at the acceptor (A) site, resulting in the activation of RelA-SpoT. This enzyme catalyzes the synthesis of guanosine 5′-diphosphate 3′-diphosphate (ppGpp), which binds to RNA polymerase and modulates its function, leading to marked changes in gene expression in the bacterial cell (1–4). Stringent control thus couples translation and transcription via the mediator ppGpp, eventually altering metabolism so that the cell is able to cope with a changing environment.

The chloroplast is a semiautonomous organelle that originated from a symbiosis between an ancestral host cell and a cyanobacterium-like photosynthetic prokaryote. Traits of the photosynthetic symbiont that are still apparent in chloroplasts include a circular DNA genome containing polycistronic gene clusters, a bacterial-type RNA polymerase consisting of a core enzyme and sigma subunit, and a translational apparatus composed of 70S ribosomes and factors of bacterial origin (5). We previously suggested the operation of ppGpp-mediated stringent control in chloroplasts on the basis of the characterization of a (p)ppGpp synthase gene in the unicellular photosynthetic eukaryote *Chlamydomonas reinhardtii* (6). Similar genes have also been identified in higher plants (7,8), and the presence of ppGpp in plant chloroplasts was recently demonstrated by high-performance liquid chromatography (9). However, the activity of the intrinsic (p)ppGpp synthase in chloroplasts has remained uncharacterized.

We have now characterized the (p)ppGpp synthase activity in chloroplast extracts derived from a higher plant. We demonstrate that a translationally active chloroplast extract contains a ribosome-associated enzyme activity that generates (p)ppGpp, and that this activity, as well as the peptide synthesis activity of the extract, is inhibited by tetracycline, a potent antibiotic that targets 70S ribosomes. These observations suggest that the (p)ppGpp synthase activity in chloroplasts, similar to that in bacteria, is dependent on the 70S ribosome.

MATERIALS AND METHODS

Plant and bacterial materials

Peas (*Pisum sativum*, cv. TOYONARI) (Sakata's seeds) were used for all experiments in this study. Seedlings were grown at 25°C under a day–night cycle of artificial light

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(8000–10 000 lux) for 16 h and darkness for 8 h. *Escherichia coli* strains W3110 (wild type) (6) and CF1678 (Δ*relA*, Δ*spoT*) (6) were used for the preparation of cellular extracts.

Preparation of organellar or cell extracts

Chloroplasts were isolated as described previously (10) from 7- to 8-day-old pea seedlings harvested 2 h into the light period. Extracts were prepared from the pea chloroplasts and *E.coli* cells as described previously (11) with minor modifications. All procedures were performed at 4°C. For the preparation of the chloroplast extract, 4 g of isolated chloroplasts were suspended in 2 ml of buffer A [50 mM Tris-acetate (pH 8.0), 15 mM magnesium acetate, 60 mM potassium acetate, 30 mM ammonium acetate, 1 mM DTT, 0.2 mM EDTA] and disrupted by 20 stokes with a Teflon pestle in a Potter-type glass homogenizer. For the preparation of the *E.coli* extract, 2 g of bacterial cells harvested from a mid-log phase culture (optical density at 600 nm, 0.7) in Luria–Bertani medium (10 g of tryptone, 5 g of yeast extract and 10 g of NaCl per liter of solution) were ground with 4 g of aluminum oxide (Nacalai Tesque, Kyoto, Japan). The cell paste was then suspended in 2 ml of buffer A. Insoluble material was removed from both the chloroplast and bacterial cell extracts by centrifugation at 15 000 *g* for 10 min. The resulting supernatants were then centrifuged at 30 000 *g* for 30 min, and the new supernatants were subjected to gel filtration on MicroSpin G-25 columns (Amersham Biosciences) that had been equilibrated with buffer A. The flow-through fractions were designated as S-30 and stored at −80°C until use. The S-30 fractions were also centrifuged at 150 000 *g* for 150 min, and both the resulting supernatants (S-150) and pellets (P-150), the latter resuspended to the original volume in buffer A, were also stored at −80°C.

To fractionate the ribosome-associating proteins from the P-150, the same pellets, used for preparing P-150, were resuspended in the original volume of buffer A containing 0.5 M NH_4Cl, and the suspensions were centrifuged at 150 000 *g* for 2.5 h. The resulting supernatants containing the released ribosome-associating factors were then dialyzed against buffer A with a dialysis membrane, Spectra/Por2 (12 000–14 000 MW cut, Spectrum Laboratories, Inc.) to remove the NH_4Cl, and were stored as 'S-150(wash)'. The resulting new pellets were briefly washed with a small amount of buffer A, resuspended in the original volume of buffer A, and stored as 'P-150(wash)'.

For further purification of the 70S ribosomal fraction, the S-30 fraction (600 µl) was layered on top of a sucrose step gradient (5 ml of 1 M and 5 ml of 0.5 M sucrose each in buffer A) and centrifuged at 150 000 *g* for 4 h at 4°C. The resulting pellet was washed twice with 1 ml of buffer A to remove the superficial brown (*E.coli*) or green (chloroplast) layer, and the remaining material was gently resuspended in 300 µl of buffer A at 4°C overnight. The resulting ribosome fraction, designated as P-150(suc), was stored at −80°C. The ribosome concentration of the various fractions was estimated spectrophotometrically by measurement of the absorbance at 260 nm and was expressed as absorbance (*A*) units per milliliter.

Detection of (p)ppGpp synthesis

ATP:GTP 5′-pyrophosphotransferase assays were performed in a final volume of 25 µl containing 2 mM ATP, 1.3 mM GTP, and either S-30 (40 *A* U/ml), P-150 (60 *A* U/ml), P-150(suc) (60 *A* U/ml), P-150(wash) (60 *A* U/ml), S-150 or S-150(wash) fraction in buffer A. The volume of the S-150 or S-150(wash) was adjusted to the corresponding volume of the P-150 or P150(wash) resuspension used in the assay. Either [α-^{32}P]GTP, [γ-^{32}P]ATP or [γ-^{32}P]GTP (each with a specific activity of 3000 Ci/mmol; Amersham Biosciences) at a concentration of 10 µCi/ml was used as a donor for radioisotope labeling. The reaction was performed for 1 h at 30°C and was stopped by the addition of 1 µl of 88% formic acid. After further addition of 10 µl of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), the mixture was agitated and then centrifuged at 10 000 *g* for 10 min at 4°C. The aqueous phase was transferred to another tube, and a 3 µl portion was spotted onto a polyethyleneimine (PEI)–cellulose thin-layer sheet (Merck). For 1D analysis, 1.5 M KH_2PO_4 was used as the chromatographic solvent, as described previously (6). Two-dimensional analysis was performed essentially as described previously (12) with solvent Tb (0.75 M Tris, 0.45 M HCl, 0.5 M LiCl) for the first dimension and solvent Sb [prepared by dissolving 74 g of $(NH_4)_2SO_4$, 0.4 g of $(NH_4)HSO_4$ and 4 g of disodium EDTA in 100 ml of H_2O] for the second dimension. Each chromatogram was exposed to a BAS-III imaging plate (Fuji Film), and the associated radioactivity was detected with a BAS-2500 analyzer (Fuji Film) and quantitated with Image Gauge version 3.41 software (Fuji Film).

Extraction of labeled nucleotides from thin-layer chromatography sheets

The nucleotides labeled with [α-^{32}P]GTP, which showed migrations similar to that of bacterial pppGpp on the two-dimensional thin-layer chromatography (2D-TLC), were extracted from the TLC sheets as described previously (13) with slight modifications. The radioactive strip corresponding to the predicted pppGpp was cut out from the sheet, and the labeled compound was extracted with 15 ml of 4 M LiCl. After filtration with Steriflip (Millipore) and neutralization with NH_4OH, the precipitates were collected by centrifugation at 4000 *g* for 10 min, washed with 95% ethanol and dissolved in 2 ml of 1 N formic acid. To the resultant solution, 99.5% ethanol (5 ml) was added, the mixture was left at −80°C for 1 h and the precipitates containing radioactive compounds were collected by centrifugation at 15 000 *g* for 20 min at 4°C. The precipitates were washed twice with 80% ethanol, air dried and suspended in 100 µl of distilled water. Insoluble materials were removed by centrifugation at 15 000 *g* for 5 min, and the soluble radioactive materials were pooled.

Characterization of the labeled nucleotide by enzyme degradation

To characterize the nucleotide predicted as pppGpp, four nucleotide-hydrolyzing enzymes were used. Three microliters of the nucleotide solution eluted from the TLC sheets were used for enzyme digestion analyses in a final volume of 30 µl, under the following conditions: myokinase (chicken muscle, Sigma) was used at 25 U/ml in 20 mM Tris–Cl (pH 7.6),

10 mM $MgCl_2$ and 5 mM AMP; bacterial alkaline phosphatase (from *E.coli* C75, Takara) was used at 0.01 U/ml in 20 mM Tris–Cl (pH 8.9) and 10 mM $MgCl_2$; snake venom phosphodiesterase I (PDE-I) (Worthington) was used at 0.1 mg/ml (3.5 U/ml) in 20 mM Tris–Cl (pH 6.5) and 10 mM $MgCl_2$; and ribonuclease T2 (RNaseT2) (Sigma) was used at 25 U/ml in 20 mM Tris–Cl (pH 6.5) and 10 mM $MgCl_2$. The enzyme digestion with the combination of snake venom PDE-I and RNaseT2 was carried out under the same buffer conditions as for the single enzyme digestion. All reactions were carried out at 37°C for 16 h. After the incubation, a 5 µl aliquot of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) was added to the reaction mixture, which was vortexed and centrifuged at 10 000 *g* for 10 min at 4°C. The aqueous phase was transferred to another tube and evaporated by using DNA120 SpeedVac system (Thermo Electron Corp.). The pellets were dissolved in 5 µl of 1 N formic acid and subjected to the 1D-TLC analysis.

Poly(U)-directed cell-free translation

To examine the effect of tetracycline on translation by chloroplast ribosomes, we used the poly(U)-directed cell-free translation system as described previously (14), but with a slight modification. Ribosome-containing fractions (20 *A* U/ml) were incubated for 10 min at 30°C in a mixture containing final concentrations of 50 mM Hepes-KOH (pH 7.6), 1.5 mM DTT, 200 mM potassium acetate, 30 mM ammonium acetate and 10 mM magnesium acetate in order to inactivate the endogenous mRNA, after which 1.2 mM ATP, 0.8 mM GTP, 0.5 mM cyclic AMP, 80 mM creatine phosphate, creatine kinase (0.25 mg/ml), rRNasin (0.5 U/ml) (Promega), poly(U) (1 mg/ml), 5 mM spermidine, L-[2,3,4,5,6-^{3}H]phenylalanine (0.025 mCi/ml) (0.1 MBq/mmol; Amersham Biosciences) and 500 µM tetracycline (all concentrations are final) were added to the mixture. The tetracycline stock solution was prepared at 12.5 mM in methanol; the same volume of methanol was added to the tetracycline-free reactions. The reaction mixtures (usually 30 µl) were then incubated at 30°C for an appropriate time. A reaction mixture without poly(U) was also incubated in parallel to confirm the low basal level of translational activity due to the residual endogenous mRNA. After incubation, 5 µl portions of each reaction mixture were applied to GF/F glass filter disks (Whatman) that had been previously soaked in 10% trichloroacetic acid (TCA) and dried. The disks were then boiled in 10% TCA for 10 min and washed twice with 10% TCA for 3 min and once with ethanol. After drying in air, the radioactivity associated with each filter disk was measured with a liquid scintillation counter.

RESULTS

Enzymatic activity of (p)ppGpp synthase in pea chloroplast extracts

To clarify whether plant chloroplasts possess a system for generating (p)ppGpp similar to that of bacteria, we attempted to measure the (p)ppGpp synthase activity in a stromal extract (S-30) prepared from pea chloroplasts. The assay was performed with [α-^{32}P]GTP, and ^{32}P-labeled mononucleotides were subsequently separated by 2D-TLC on PEI-cellulose plates and visualized by autoradiography. Each spot was identified on the basis of its retardation factor (R_f) as described previously (12), and the positions of ppGpp and pppGpp were verified by comparison with the 2D-TLC patterns obtained by the parallel examination of an *E.coli* cell extract.

The S-30 fractions prepared from isolated pea chloroplasts (Figure 1A) and a wild-type strain (W3110) of *E.coli* (Figure 1B) generated signals corresponding to ppGpp and pppGpp. In contrast, no such signals were observed with the S-30 fraction derived from a *relA-spoT* mutant (CF1678) of *E.coli* (Figure 1C), indicative of the complete loss of (p)ppGpp-generating activity (15). In the reactions mediated by all three extracts, the [α-^{32}P]GTP was converted to GDP and GMP and ^{32}P-labeled ATP and ADP were also formed, indicative of the extensive mononucleotide metabolism in these extracts.

To further examine the exchange of the phosphate moiety among mononucleotides in the chloroplast extract, we also performed the (p)ppGpp synthase activity assay with [γ-^{32}P]ATP or [γ-^{32}P]GTP as the labeled nucleotide. The results obtained with [γ-^{32}P]ATP were similar to those observed with [γ-^{32}P]GTP, in which the *E.coli* W3110 extract produced more ppGpp than pppGpp (Figure 1F), whereas the chloroplast extract generated more pppGpp than ppGpp (Figure 1E). Again, neither ppGpp nor pppGpp was produced by the *E.coli* CF1678 extract (Figure 1G). Labeled GTP, GDP and ADP were also generated by all three extracts, indicative of the presence of nucleoside diphosphate kinase, guanylate kinase and adenylate kinase. The 2D-TLC patterns obtained with [γ-^{32}P]GTP as the labeled nucleotide were virtually identical to those obtained with [γ-^{32}P]ATP for all three extracts (Figure 1I–K). The appearance of ^{32}P-labeled ATP, ADP and GDP was indicative of the incorporation of the γ-phosphate of GTP into these nucleotides. Indeed, the generation of ^{32}P-labeled orthophosphate by each extract with each of the three labeled substrates suggested the occurrence of dephosphorylation coupled with *de novo* synthesis of each labeled nucleotide.

These observations thus demonstrated the presence of (p)ppGpp synthase activity in a plant chloroplast extract. However, given the highly active nucleotide metabolism apparent in the extract, it remained unclear whether the (p)ppGpp synthesis observed was attributable to the ATP pyrophosphate transferase activity of the pea RelA-SpoT homolog.

Characterization of the labeled pppGpp-like nucleotide

To characterize the chemical structure of the ^{32}P-labeled nucleotide assumed to be pppGpp in Figure 1A, we next performed enzyme digestion assays of the labeled nucleotide with hydrolyzing enzymes, such as PDE-I, RNaseT2, alkaline phosphatase and myokinase. The nucleotide labeled with [α-^{32}P]GTP was purified from 2D-TLC plates as described in the Materials and Methods, and was subjected to the enzyme assays. The resultant labeled moieties generated by the enzyme treatment were analyzed by 1D-TLC.

As shown in Figure 2, PDE-I digestion resulted in a labeled moiety at the Gp_3 position, indicating the generation of pGpp, since PDE-I specifically hydrolyzes the α-β-pyrophosphate

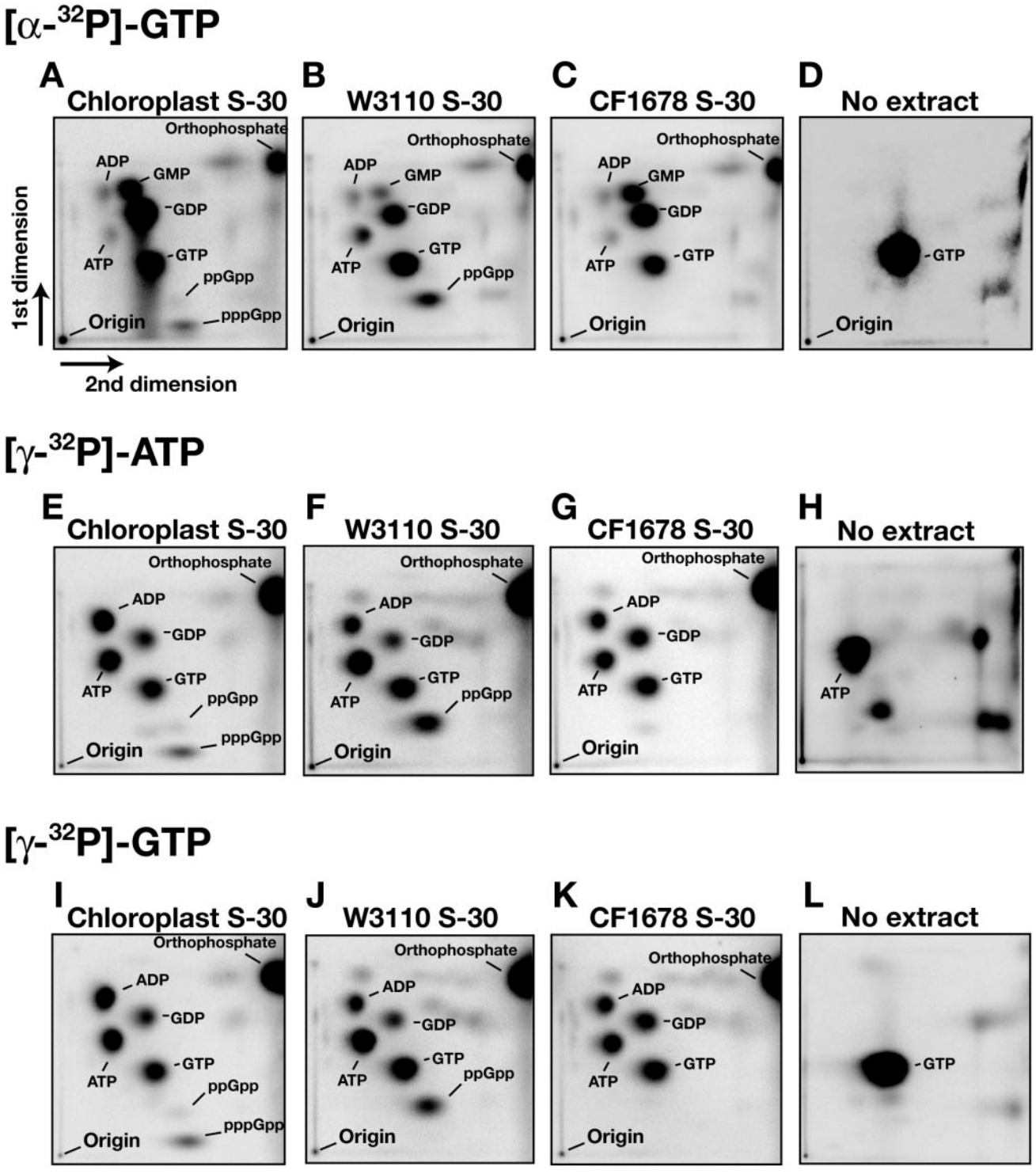

Figure 1. Assay of the S-30 fractions of pea chloroplasts and *E.coli* cells for (p)ppGpp synthase activity. The S-30 fractions of pea chloroplasts (**A**, **E**, **I**) or of *E.coli* strains W3110 (**B**, **F**, **J**) or CF1678 (**C**, **G**, **K**) were incubated with [α-^{32}P]GTP (A, B, C), [γ-^{32}P]ATP (E, F, K) or [γ-^{32}P]GTP (I, J, K), after which ^{32}P-labeled nucleotides were separated by 2D-TLC on PEI-cellulose sheets and visualized by autoradiography. The origin and the positions of spots corresponding to ADP, ATP, GMP, GDP, GTP, ppGpp, pppGpp and orthophosphate are indicated. Data are representative of experiments performed with three independent chloroplast or bacterial extracts. As a negative control, the 2D-TLC profile of each nucleotide used for labeling is shown on the right end of each panel as 'no extract' (**D**, **H**, **L**).

linkage of nucleotide 5′-polyphosphates (12,13). On the other hand, digestion with RNaseT2, which hydrolyzes the α-β-pyrophosphate linkage of nucleoside 3′-polyphosphates (12), resulted in a labeled moiety at the Gp_4 position, indicating the conversion from pppGpp to pppGp, as described previously (12,13). The combination of PED-I and RNaseT2 resulted in a labeled moiety at the Gp_2 position, which is presumed to be pGp, showing good agreement with the results from the

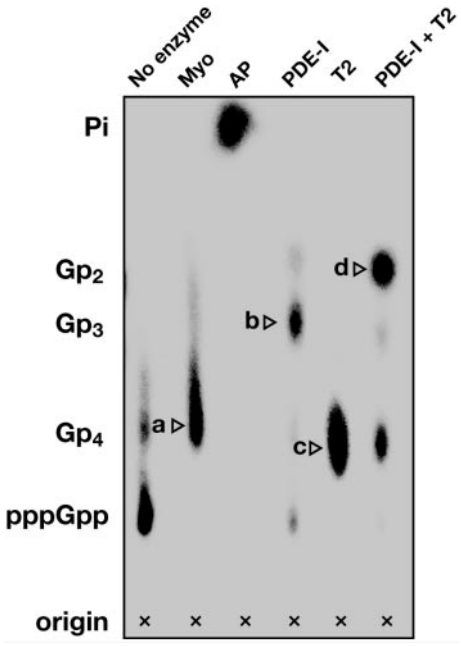

Figure 2. Characterization of the labeled pppGpp-like nucleotide by nucleotide-hydrolyzing enzymes. The radioactive compounds corresponding to putative pppGpp were extracted from PEI-cellulose sheets and subjected to enzyme digestion analysis. Abbreviations for enzymes are indicated as: Myo, myokinase; AK, bacterial alkaline phosphatase; PDE-I, snake venom phosphodiesterase type I; T2, ribonuclease T2; and PDE-I + T2, combination of the phosphodiesterase and the ribonuclease. Enzyme reactions were performed as described in the Materials and Methods. After the digestion, ^{32}P-labeled compounds were separated by 1D-TLC as described previously (6) and visualized by autoradiography. Four detected nucleotide spots (a–d) are indicated in the picture with arrowheads. Pi, Gp_2, Gp_3 and Gp_4 indicate orthophosphate, guanosine diphosphate, guanosine triphosphate and guanosine tetraphosphate, respectively. These positions were determined by co-chromatography of similarly labeled nucleotides prepared from the *E.coli* W3110 extract (13).

pppGpp digestion by these enzymes, as described previously (13). Treatment of the nucleotide with myokinase, which may transfer the 5′-γ-phosphate from nucleotide 5′-polyphosphates to AMP (16), also resulted in a labeled moiety at the Gp_4 position, indicating the generation of ppGpp by the removal of the 5′-γ-phosphate from pppGpp. The mobility of this material, presumed to be ppGpp, in fact slightly differs from the putative pppGp, the resultant of the RNaseT2 treatment, on the 1D-TLC (Figure 2). In addition, alkaline phosphatase digestion of the nucleotide resulted in an orthophosphate spot in the 1D-TLC, indicating that the nucleotide is not a cyclic-phosphonucleotide (12).

These results thus demonstrate that the examined nucleotide is a guanosine 5′-diphosphate 3′-diphosphate (pppGpp).

Association of chloroplast (p)ppGpp synthase activity with 70S ribosomes

We next investigated whether the (p)ppGpp synthase activity detected in the S-30 chloroplast extract is associated with the 70S ribosomes present in this fraction. The ribosomes were purified by centrifugation of the S-30 extract at 150 000 *g*, either directly or through a sucrose gradient, yielding P-150 and P-150(suc) fractions, respectively (Figure 3A). The P150(suc) fraction of wild-type *E.coli* cells mediated the synthesis of both ppGpp and pppGpp from [α-^{32}P]GTP (Figure 3B), suggesting that the (p)ppGpp synthase activity attributable to RelA is associated with 70S ribosomes (11,17). In contrast, the P-150(suc) fraction of pea chloroplasts did not generate a detectable level of ppGpp or pppGpp upon incubation with [α-^{32}P]GTP, whereas the chloroplast P-150 fraction generated a clear signal corresponding to pppGpp and a less pronounced signal corresponding to ppGpp (Figure 3B).

To further examine the association of the (p)ppGpp synthase activity with the chloroplast 70S ribosome, we performed the enzyme assay with the P-150(wash) fraction, in which the ribosome was treated with 0.5 M NH_4Cl to remove the ribosome-associating factors, and with the S-150(wash) fraction containing released factors from the washed-ribosomes. We observed that the (p)ppGpp synthase activity in the P-150(wash) was substantially reduced (83%) by the high-salt treatment of the ribosome, and that the activity was recovered to ∼50% of that seen in the P-150 by supplementation with the S-150(wash), which did not generate a detectable level of ppGpp or pppGpp by itself (Figure 3B). We also examined the pppGpp synthase activity of the P-150(suc) supplemented with the S-150(wash), but no restoration of the activity could be observed (data not shown).

These observations suggest that the (p)ppGpp synthase activity of pea chloroplasts is stimulated by 70S ribosomes, and the responsible enzyme is associated weakly with 70S ribosomes. It seems that the pppGpp synthase is dissociated from the ribosomes by the treatment of 70S ribosomes with a high concentration of salts, like the bacterial RelA protein (17,18), or by the centrifugation through the sucrose gradient.

Inhibition of translational activity of chloroplasts by tetracycline

Tetracycline potently inhibits bacterial protein synthesis by blocking the entry of aminoacylated tRNA into the A site of the 70S ribosome (19). As far as we are aware, the effect of this antibiotic on translation in the chloroplasts of higher plants, however, has not been described. We therefore investigated whether tetracycline inhibits the peptide elongation activity of the S-30 fraction of pea chloroplasts, with the S-30 fraction of wild-type *E.coli* as a positive control. A poly(U)-directed translation assay with ^{3}H-labeled phenylalanine revealed that tetracycline (500 μM) inhibited the peptide elongation activities of the S-30 fractions of both pea chloroplasts (Figure 4A) and *E.coli* cells (Figure 4B).

We further examined the dose effects of tetracycline on the inhibition of the peptide elongation activity in the chloroplast and bacterial extracts. As shown in Figure 4C and D, the peptide elongation activities in both extracts were inhibited in a dose-dependent manner, and the IC_{50} values of tetracycline in the *E.coli* and chloroplast extracts were calculated as approximately 37 and 135 μM, respectively.

To provide support for our *in vitro* observation, we compared the structural features of plastid and bacterial ribosomes. Characterization of the crystal structure of the complex of the 30S subunit of the bacterial ribosome with tetracycline (20) as well as biochemical analyses by chemical cross-linking assays (21–24) have identified the sites of the small ribosomal subunit that interact with tetracycline. On the other hand, bacterial

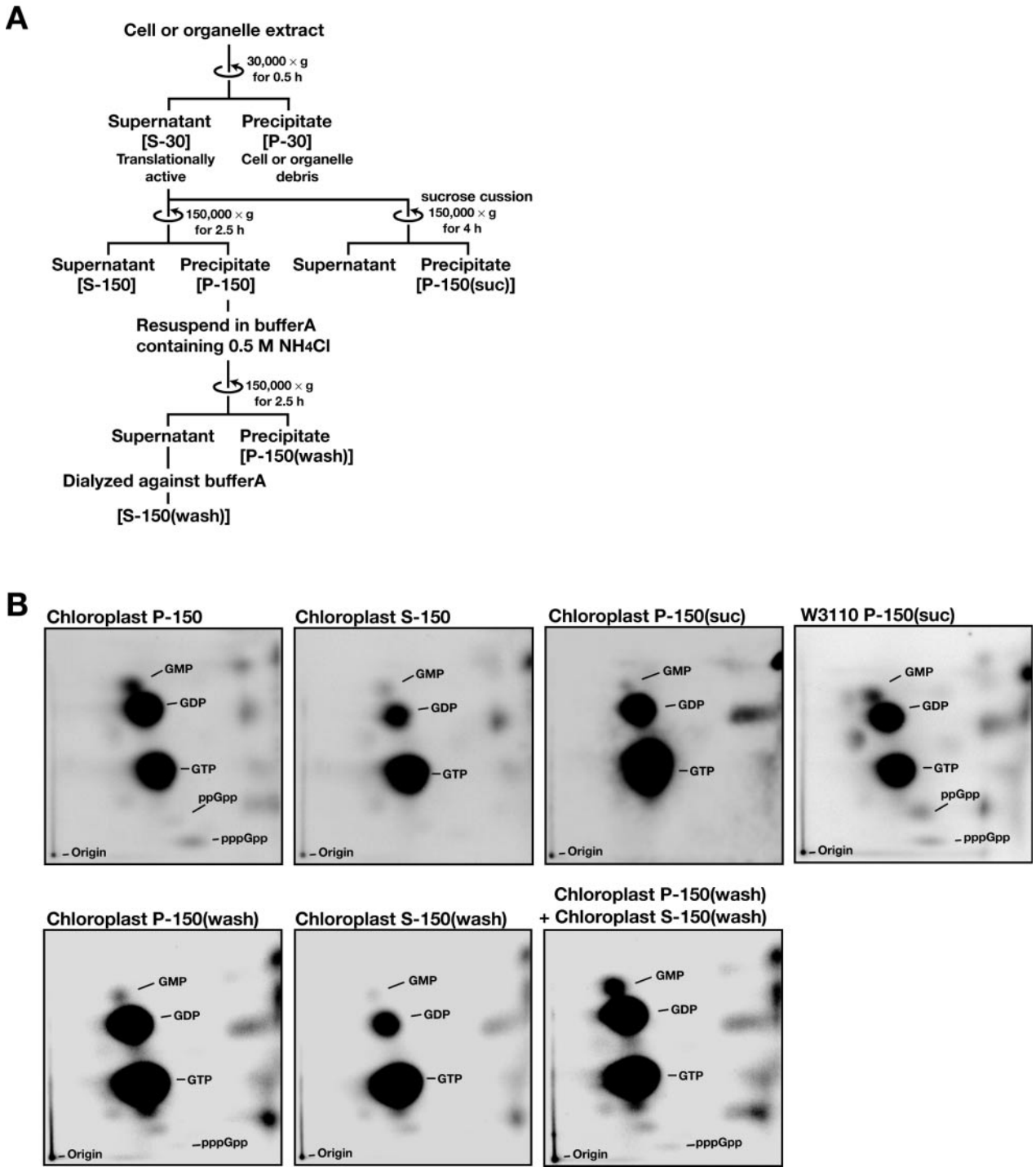

Figure 3. Association of (p)ppGpp synthase activity with 70S ribosomes in pea chloroplasts. (**A**) Schematic representation of the procedure for fractionation of pea chloroplast and bacterial cell extracts. (**B**) Assay of the chloroplast and *E.coli* W3110 P-150(suc) fractions for (p)ppGpp synthase activity with [α-^{32}P]GTP as substrate. The chloroplast P-150, S-150, P-150(wash), and S-150(wash) fractions, and the reconstituted fraction consisting of chloroplast P-150(wash) and S-150(wash) were examined. The reaction mixtures were subjected to 2D-TLC and autoradiography. Data are representative of experiments performed with three independent chloroplast or bacterial extracts. Relative intensities of the pppGpp spots appeared from the chloroplast fractions were: P-150, 100; S-150, 2.6; P-150(suc), 4.6; P-150(wash), 17.2; S-150(wash), 5.8; P-150(wash) + S-150(wash), 49.9. Spot intensities were quantitated based on the densitometric analysis as described in Materials and Methods.

genetic studies on tetracycline-resistant mutants have revealed that specific mutations in the 16S rRNA can restore the growth defect caused by the antibiotic (25,26). The components of chloroplast ribosomes have also been characterized at the nucleotide sequence level (27). Comparisons of the 16S rRNA nucleotide sequences and the predicted amino acid sequences of ribosomal proteins between bacteria and chloroplasts of higher plants have revealed overall similarities

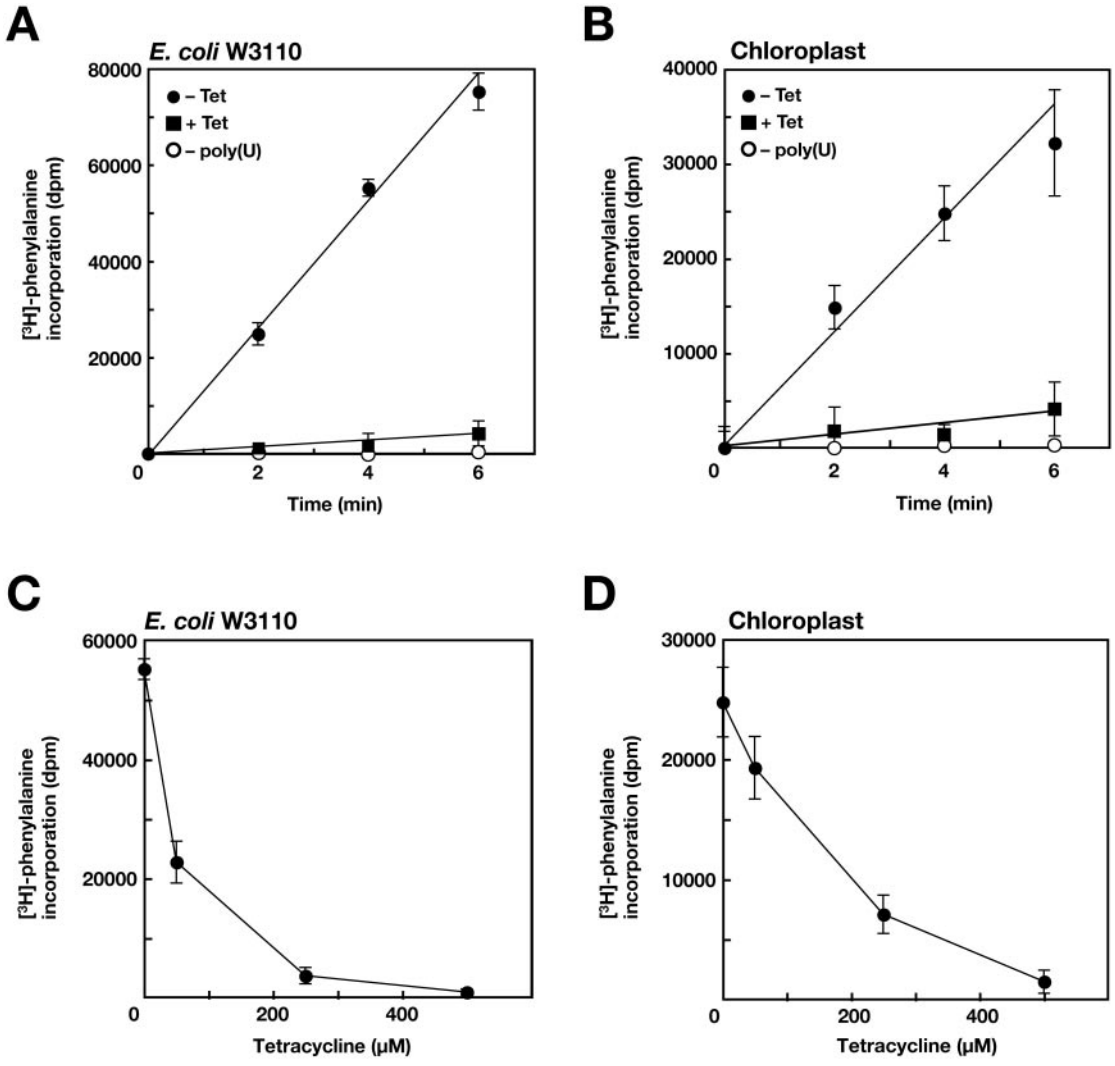

Figure 4. Inhibition of the peptide elongation activity of pea chloroplasts by tetracycline. The peptide elongation activity of the S-30 fractions of pea chloroplasts and *E.coli* W3110 cells was determined in the absence (closed circles) or in the presence (closed squares) of 500 μM tetracycline with a poly(U)-dependent *in vitro* translation assay (**A** and **B**), and the dose effect of tetracycline on the translation activity was examined in the presence of several concentrations (0, 50, 250 and 500 μM) of tetracycline (**C** and **D**). The reaction mixture contained 0.025 mCi/ml of L-[2,3,4,5,6-^{3}H]phenylalanine, with a specific radioactivity of 108 Ci/mmol, and the incorporation of [^{3}H]phenylalanine into poly-phenylalanine is indicated by disintegradations per minute (dpm). One dpm is equivalent to 8.34×10^4 pmol phenylalanine per ml in the reaction. Data represent the incorporation of [^{3}H]phenylalanine into TCA-precipitable material during the incubation of reaction mixtures for the indicated times. The activity of a reaction mixture without poly(U) (open circles) was also determined as a negative control. Results are representative of experiments performed with three independent chloroplast or bacterial extracts, and SDs are indicated by bars. The dose-effect analysis was performed by measurement of the incorporated tritium at 4 min after the start of the reaction.

(27–29). Our analysis showed that the nucleotides in the tetracycline-interacting regions of bacterial 16S and 23S rRNAs are highly conserved in the corresponding chloroplast rRNAs (Table 1). A crystallographic analysis of the structure of the complex of the 30S subunit of the bacterial ribosome with tetracycline identified six tetracycline-binding sites (20). The binding of tetracycline to the Tet-1 region of the bacterial 16S rRNA has been proposed to affect the structure of the nearby A site, resulting in the inhibition of protein synthesis (20,30). The nucleotides that form the Tet-1 site of *E.coli* 16S rRNA (A964–G966, G1053, C1054, A1196–G1198) are completely conserved in the 16S rRNA of pea chloroplasts (Table 1). Moreover, G1058 of bacterial 16S rRNA, the site of a mutation (G1058C) that confers tetracycline resistance (31), is also conserved in chloroplast 16S rRNA (Table 1). Thus, the inhibitory effect of tetracycline on the protein synthetic activity of pea chloroplasts is likely to be attributable to the specific interaction of the antibiotic with the 70S ribosome.

Inhibition of chloroplast (p)ppGpp synthase activity by tetracycline

Tetracycline also inhibits (p)ppGpp synthase activity in *E.coli* by blocking the entry of deacylated tRNA into the A site of the ribosome (19). Given that our results showed that tetracycline inhibits the peptide elongation activity of chloroplasts, presumably by a mechanism similar to that by which it achieves this effect in bacteria, we next investigated whether the (p)ppGpp synthase activity of chloroplast extracts is also

Table 1. Tetracycline-binding sites of bacterial rRNA and their conservation in pea chloroplast rRNA

rRNA	Tetracycline-binding site of bacterial rRNA[a]	Equivalent nucleotides in pea chloroplast rRNA[b]	Comment	Reference
16S	A892, U1052, C1054	All conserved	Tetracycline protection against chemical footprinting	(21)
16S	G693, G890, G1330, G1338	All conserved	Tetracycline-nucleotide cross-linking	(23)
16S	U244, G894, C967, C1400, C1402, C1501	All conserved	Tetracycline inhibition of nucleotide–nucleotide cross-linking (U244 × G894, C967 × C1400, C1402 × C1501)	(22)
16S	G1058	Conserved	Tetracycline-resistant G1058C mutation	(31)
16S	C936, C948	All conserved	Tetracycline-nucleotide cross-linking	(24)
16S	A964–G966, G1053, C1054, A1196–G1198	All conserved	Crystal structure, Tet-1 site	(20)
16S	C1162–G1164, C1172–G1174	Conserved except A1163G and U1173C	Crystal structure, Tet-3; differences also exist in *Thermus thermophilus* 16S rRNA (A1163C, U1173G)[a]	(20)
16S	G941, G942, C1342, G1343, A1349–U1351	All conserved	Crystal structure, Tet-4	(20)
16S	U244–G247, G894–C896	Conserved except G895U and C896U	Crystal structure, Tet-5	(20)
16S	A937, A938, C1378–U1380	All conserved	Crystal structure, Tet-6	(20)
16S	A965, G966, A967	All conserved	Tetracycline-resistant AGA965-967UUC mutation; differences exist in *Helicobacter pylori* 16S rRNA (U965A, C967A)[a]	(25,26)
23S[b]	G2505, G2576, G2608	All conserved	Tetracycline-nucleotide cross-linking	(23)

[a]Bases of rRNA are numbered according to the *E.coli* 16S and 23S rRNA sequences.
[b]Pea chloroplast 16S and 23S rRNA sequences, accession nos. X51598 and X55033.

inhibited by tetracycline. The (p)ppGpp synthase activity of the S-30 fraction of pea chloroplasts was indeed inhibited by tetracycline (Figure 5A and B), indicating that (p)ppGpp synthesis is also dependent on 70S ribosomal function in chloroplasts. The dose-response curves for the tetracycline inhibition of (p)ppGpp synthesis in the pea chloroplast fraction (Figure 5D) and in the bacterial extract (Figure 5C) were very similar to those observed for the inhibition of the peptide elongation activities (Figure 4D and C).

DISCUSSION

Here, we have shown that, similar to bacteria, chloroplasts of a higher plant possess a system for generating (p)ppGpp. Extracts of pea chloroplasts were thus found to contain (p)ppGpp synthase activity similar to that within extracts of wild-type *E.coli* cells. A recent report demonstrated that the chloroplast RNA polymerase activity is inhibited in the presence of ppGpp *in vitro*, suggesting the existence of a bacterial-type stringent response (9). Our observations therefore further implicate (p)ppGpp synthase activity in the genetic system of higher plant chloroplasts. Enzyme activity assays performed with three different ^{32}P-labeled mononucleotides also revealed an extensive pattern of nucleotide metabolism in chloroplast extracts, suggesting the presence in this organelle of enzymes, such as adenylate kinase, guanylate kinase and nucleoside diphosphate kinase. The generation of [^{32}P]orthophosphate from [α-^{32}P]GFP, [γ-^{32}P]GTP and [γ-^{32}P]ATP by chloroplast extracts was also consistent with the broad exchange of phosphate moieties among mononucleotides in this organelle. The presence of adenylate kinase in maize chloroplasts (32) and of nucleoside diphosphate kinase in pea chloroplasts (33) has been described previously.

The S-30 fraction of pea chloroplasts generated more pppGpp than ppGpp. In contrast, Takahashi *et al*. (9) showed that intact pea chloroplasts accumulated ppGpp in larger amounts than they did pppGpp *in vivo*. It is possible that this discrepancy is attributable to differences in nucleotide metabolism between the *in vivo* and *in vitro* conditions. GTP is rapidly dephosphorylated to GDP by the GTPase activities associated with the translational apparatus. The efficiency of GDP metabolism might, however, differ between the *in vivo* and *in vitro* conditions of the two studies as a result of the loss of certain chloroplast components, such as thylakoid membrane-bound enzymes, during preparation of the S-30 fraction. Determination of the precise mechanism of (p)ppGpp synthesis by chloroplasts will require biochemical analyses with purified components, as was the case with the *E.coli* system (19).

Although the P-150(suc) fraction of wild-type *E.coli* cells contained (p)ppGpp synthase activity, the corresponding fraction of pea chloroplasts did not. This result is consistent with the previous observation that a similarly prepared ribosomal fraction of *C.reinhardtii* chloroplasts did not possess (p)ppGpp synthase activity (34). These various fractions lack chloroplast components that associate weakly or not at all with ribosomes. In contrast, we did detect (p)ppGpp synthase activity in the P-150 fraction of pea chloroplasts, which was prepared by centrifugation at the same speed as that used for the preparation of P-150(suc) but without the sucrose gradient. The supernatant (S-150) corresponding to the P-150 fraction of pea chloroplasts did not contain (p)ppGpp synthase activity. Supplementation of P-150 with S-150 did not increase or inhibit the (p)ppGpp synthase activity of the former (data not shown), indicating that the (p)ppGpp synthase activity co-exists with the 70S ribosome in the P-150 fraction. Moreover, we observed that washing the ribosomes with

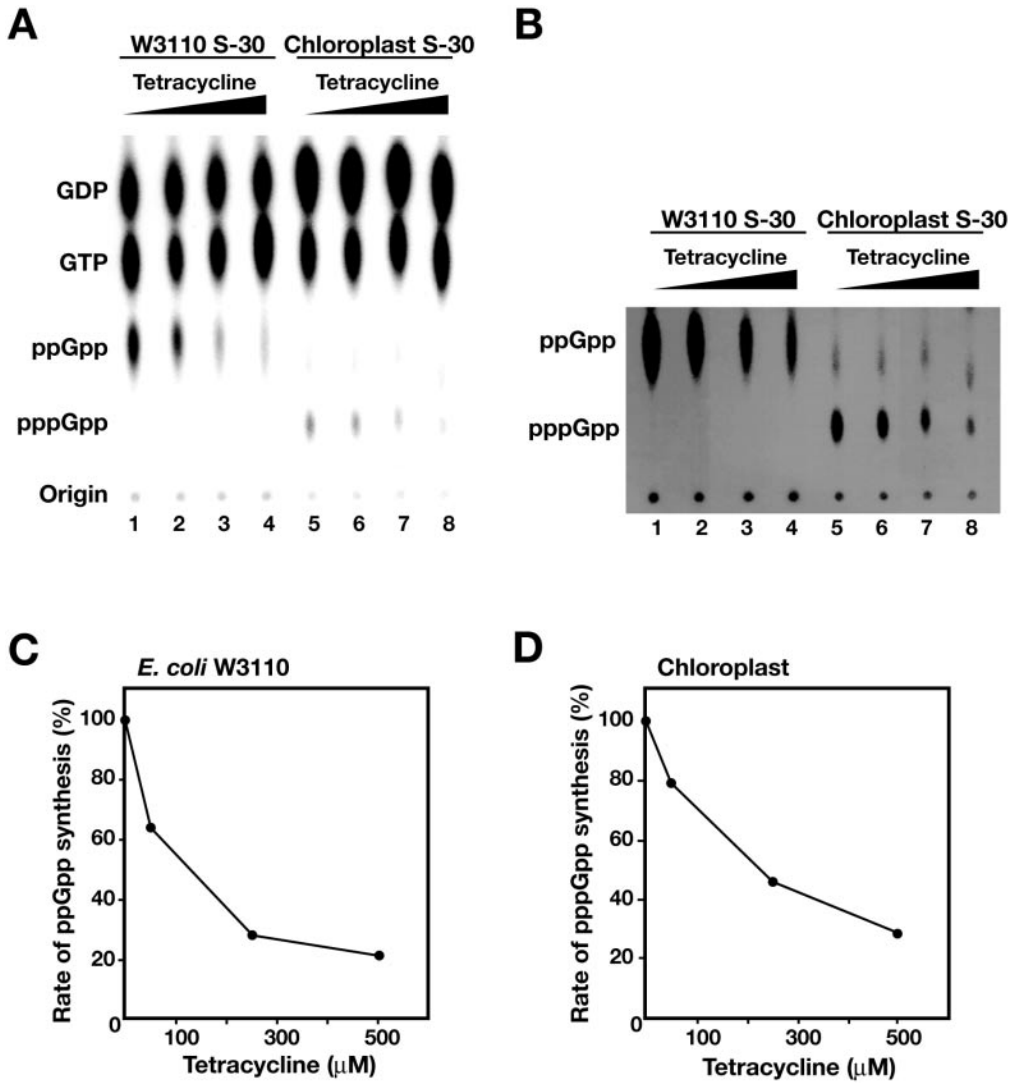

Figure 5. Inhibition by tetracycline of the (p)ppGpp synthase activity of pea chloroplast extract. (**A**) The S-30 fractions of pea chloroplasts and *E.coli* W3110 cells were assayed for (p)ppGpp synthase activity with [α-^{32}P]GTP as substrate in the presence of tetracycline at concentrations of 0 μM (lanes 1 and 5), 50 μM (lanes 2 and 6), 250 μM (lanes 3 and 7) or 500 μM (lanes 4 and 8). Labeled nucleotides were separated by 1D-TLC on PEI-cellulose and visualized by autoradiography (12 h exposure). (**B**) Longer exposure (36 h) of the TLC plate shown in (A). (**C** and **D**) The intensities of the ppGpp spots obtained with the S-30 fraction of *E.coli* (C) or of the pppGpp spots obtained with the S-30 fraction of pea chloroplasts (D) were measured for the autoradiogram shown in (B). Data are expressed relative to the corresponding values for the incubations performed in the absence of tetracycline, and are representative of experiments performed with three independent chloroplast or bacterial extracts.

high salt substantially reduces the (p)ppGpp synthase activity of the P-150(wash) fraction, and that supplementation of the P-150(wash) with the S-150(wash) fraction, containing components removed from the ribosome, clearly enhances the (p)ppGpp synthase activity. We therefore conclude that the (p)ppGpp synthase activity of pea chloroplasts is weakly associated with 70S ribosomes.

Most of the ribosomal proteins and rRNA molecules of chloroplasts are similar to the corresponding components of *E.coli* ribosomes at the amino acid or nucleotide sequence level, respectively (27–29). Certain ribosome-targeting antibiotics, including chloramphenicol (35), streptomycin (36) and spectinomycin (37), also inhibit the translational activity of plant chloroplasts, consistent with a conservation of structural features between the 70S ribosomes of bacteria and chloroplasts. We have now shown that tetracycline also inhibits the peptide elongation activity in extracts of pea chloroplasts. Our observation that this antibiotic does not appear to affect the growth of tobacco or rice plants when added to soil or growth medium (Y.T. *et al*., unpublished data) suggests that tetracycline may have difficulty in crossing cellular membranes or be subject to detoxification in intact plants. In addition to its effect on translation, tetracycline inhibited the (p)ppGpp synthase activity of pea chloroplast extracts *in vitro*. Furthermore, a comparison of the structures of bacterial and chloroplast rRNA molecules revealed that the putative tetracycline-binding regions of bacterial 16S rRNA are well conserved in plastid 16S rRNA. The inhibitory effects of tetracycline on protein synthesis and (p)ppGpp synthase activity in chloroplast extracts suggest that the enzymatic function of the chloroplast (p)ppGpp synthase is stimulated by 70 ribosomes, as in the bacterial system.

In conclusion, we have shown that pea chloroplasts possess (p)ppGpp synthase activity, and that this activity is associated with 70S ribosomes and sensitive to tetracycline. We propose not only that plant chloroplasts possess a bacterial-type (p)ppGpp synthase (6,8) but also that the function of this enzyme is coupled with the translation apparatus, like the corresponding enzymes in the bacterial system.

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