Diversity in Guanosine 3′,5′-Bisdiphosphate (ppGpp) Sensitivity among Guanylate Kinases of Bacteria and Plants*

Received for publication, November 12, 2013, and in revised form, March 20, 2014. Published, JBC Papers in Press, April 10, 2014, DOI 10.1074/jbc.M113.534768

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Background: The ppGpp signaling system is operative in plant chloroplasts and bacteria.

Results: Chloroplast and cytosolic guanylate kinases (GKs) of plants are sensitive and insensitive to ppGpp, respectively, whereas bacterial GKs show diversity in ppGpp sensitivity.

Conclusion: GTP biosynthesis in chloroplasts is controlled by ppGpp.

Significance: Identification of the targets of ppGpp should provide insight into biological processes regulated by this nucleotide.

The guanosine 3′,5′-bis[diphosphate (ppGpp) signaling system is shared by bacteria and plant chloroplasts, but its role in plants has remained unclear. Here we show that guanylate kinase (GK), a key enzyme in guanine nucleotide biosynthesis that catalyzes the conversion of GMP to GDP, is a target of GK, a key enzyme in guanine nucleotide biosynthesis which is also a cytosolic enzyme. These observations suggest that ppGpp plays a pivotal role in the regulation of GTP biosynthesis in chloroplasts through specific inhibition of GK activity, with the IC50 for inhibition of GK activity by ppGpp being estimated as that of GK from bakers’ yeast, which is also a cytosolic enzyme. Our biochemical characterization of GK enzymes has thus revealed a novel target of ppGpp in chloroplasts and has uncovered diversity among bacterial GKs with regard to regulation by ppGpp.

The hyperphosphorylated guanine ribonucleotide guanosine 3′,5′-bis[diphosphate (ppGpp) is known as the global regulator in bacteria because it regulates the functions of multiple protein-based molecular machines in response to various stressors (1–4). The ppGpp signaling system also plays a central role in microbial persistence and has thus been considered as a potential drug target for the control of bacteria responsible for infectious diseases (5). This signaling system is also operative in plant chloroplasts, with vascular plants having been found to express several distinct types of ppGpp synthetase (6–10). One of these enzymes, CRSH (Ca2+ -activated RelA/SpoT homolog), is a plant-specific Ca2+-dependent ppGpp synthetase, and its function is essential for plant reproduction (11).

We have previously shown that the chloroplast translation system is negatively regulated by ppGpp in vitro (12), and others have suggested that chloroplast RNA polymerase is a target of ppGpp by demonstrating a physical interaction between a ppGpp analog and the polymerase (13). It remains unclear, however, whether translation and transcription are primary targets of ppGpp in chloroplasts, given that the estimated inhibitory concentrations for ppGpp of ~250 μM (12) and >1 mM (6), respectively, are relatively high.

Targets of ppGpp in bacteria have been found to include RNA polymerase, translation factors, DNA primase, and enzymes of RNA biosynthesis (3, 9). Guanylate kinase (GK) was recently identified as a key target of ppGpp in Bacillus subtilis, with the IC50 for inhibition of GK activity by ppGpp being estimated at ~30 μM, which is within the concentration range of physiological ppGpp fluctuation in this bacterium (14). Plants possess two distinct GK enzymes, cytosolic GK (GKc) and plastid/mitochondrial GK (GKpm) (15, 16). Analysis of rice mutants has revealed that both GKc and GKpm are essential for plant growth and development (16). Plant cells possess purine biosynthetic pathways that differ from those in other euca-

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB916335 and AB916336.

* This work was supported by a Grant-in-Aid for Scientific Research 24570054 (to Y. T.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and a Grant-in-Aid for JSPS Fellows (to Y. Nomura) from the Japan Society for the Promotion of Science.

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2 The abbreviations used are: ppGpp, guanosine 3′,5′-bis[diphosphate; GK, guanylate kinase; GKc, cytosolic GK; GKpm, plastid/mitochondrial GK; Tribine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethy]glycine.

MAY 30, 2014 • VOLUME 289 • NUMBER 22

ASBMB

JOURNAL OF BIOLOGICAL CHEMISTRY

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**FIGURE 1. Purine biosynthetic pathways in plant cells and bacteria.** The following abbreviations are used: PRPP, 5-phosphoribosyl-1-pyrophosphate; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; FGAM, formylglycinamidine ribonucleotide; AIR, 5-aminomimidazole ribonucleotide; CAIR, 4-carboxyl aminoimidazole ribonucleotide; FAICAR, N-succinyl-5-aminomimidazole-4-carboxamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, 5-formaminoimidazole-4-carboxamide ribonucleotide; XMP, xanthosine monophosphate; AdS, adenylosuccinate; IMPD, IMP dehydrogenase; AdSS, adenylosuccinate synthase; GMPS, GMP synthase. All plant homologs of enzymes are present in the genome database (Table 1). Broken arrows indicate unidentified or hypothetical routes for membrane transport.

otic species (17) (Fig. 1, Table 1). Whereas the ATP and GTP pathways diverge at IMP in all living organisms, the enzymes at the branch point are thought to localize to different compartments in plant cells, with adenylosuccinate synthase localization to plastids and likely also to mitochondria and IMP dehydrogenase being restricted to the cytosol (17–19). In contrast to ATP biosynthesis in organelles, two distinct sets of enzymes (GK and nucleoside diphosphate kinase) that are required for the conversion of GMP to GTP are present in both chloroplasts and cytosol (20, 21). As a result of this disjunction at GMP, GK appears to be responsible for the first step of GTP biosynthesis in chloroplasts (Fig. 1).

We have now examined the effects of ppGpp on GK catalytic activity in vitro. We found that the organellar GK of rice (Oryza sativa) (OsGKpm), pea (Pisum sativum) (PsGKpm), and Ara-

**TABLE 1**

| Enzyme                                | Subcellular localization |
|----------------------------------------|--------------------------|
| Adenylosuccinate synthase              | Organelle/Cytosol        |
| Adenylate kinase                       |                          |
| IMP dehydrogenase                      |                          |
| GMP synthase                          |                          |
| Guanylate (GMP) kinase                 |                          |
| Nucleoside diphosphate kinase          |                          |

**EXPERIMENTAL PROCEDURES**

Plasmid Construction—DNA fragments encoding a truncated form (ND92) of organellar GK of rice (OsGKpm) and cytosolic GK of *S. cerevisiae* (ScGK) were cloned into the expression vector pET28a(+) (Novagen, Madison, WI) as described previously (16). Primers for PCR are listed in Table 2. For construction of expression vectors for ND45 and ND73 forms of OsGKpm, the corresponding DNA fragments were amplified by PCR from cDNA of *O. sativa* (8) with the primers OsGKpm-Δ1-forward and OsGKpm-reverse for ND45, and OsGKpm-Δ2-forward and OsGKpm-reverse for ND73. The PCR products were cloned directly into the pTA2 vector with the use of a Target Clone Kit (Toyota, Osaka, Japan). The resulting plasmids were then digested with NdeI and BamHI, and the released gene fragments were cloned into the corresponding sites of pET28a(+) OsGKpm (ND92). A DNA fragment encoding cytosolic GK of rice (OsGKc, GenBank accession no. AB267729) was amplified by PCR from cDNA of *O. sativa* with primers OsGKc-forward and OsGKc-reverse and cloned into pTA2. A DNA fragment encoding a truncated form (ND37) of organellar GK of *A. thaliana* (AtGK3, AF378877) was amplified by PCR from cDNA of *A. thaliana* (22) with primers AtGK3-forward-1 and AtGK3-reverse-1. The amplified DNA fragment was used as a template for a second PCR with primers AtGK3-forward-2 and AtGK3-reverse-2, the product was cloned directly into pTA2, and the NcoI-NdeI
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List of PCR primers

| Primer name       | Primer sequence (5’ to 3’) |
|-------------------|----------------------------|
| OsGKpkm-Δ1-forward| ttccatgtctctcggcttgccacactct |
| OsGKpkm-Δ2-forward| ttccatgtgggggctgctggccagagtt |
| OsGKpkm-forward   | aaagattccctctatcccccctagaca |
| OsGKc-forward     | tttcatggggcagccatcacatcatcacagcagccgcatggtagggtaaagggagtctcgg |
| OsGKc-reverse     | aaagattccctctatcccccctagaca |
| BsGK-forward      | tttcatggggcagccatcacatcatcacagcagccgcatggtagggtaaagggagtctcgg |
| BsGK-reverse      | aaagattccctctatcccccctagaca |
| EcGK-forward      | tttcatggggcagccatcacatcatcacagcagccgcatggtagggtaaagggagtctcgg |
| EcGK-reverse      | aaagattccctctatcccccctagaca |
| SeGK-forward      | tttcatggggcagccatcacatcatcacagcagccgcatggtagggtaaagggagtctcgg |
| SeGK-reverse      | aaagattccctctatcccccctagaca |
| PsGKpm-forward-1  | atggatgtcagcttctggagaag |
| PsGKpm-forward-2  | tttcatatggcttgcttgcttggag |
| PsGKpm-reverse-1  | ttagctagcttgcttgcttggag |
| PsGKpm-reverse-2  | tttagctagcttgcttgcttggag |
| PsGKc-forward-1   | tttagctagcttgcttgcttggag |
| PsGKc-forward-2   | tttagctagcttgcttgcttggag |
| PsGKc-reverse-1   | tttagctagcttgcttgcttggag |
| PsGKc-reverse-2   | tttagctagcttgcttgcttggag |
| AtGK3-forward-1   | tttagctagcttgcttgcttggag |
| AtGK3-forward-2   | tttagctagcttgcttgcttggag |
| AtGK3-reverse-1   | tttagctagcttgcttgcttggag |
| AtGK3-reverse-2   | tttagctagcttgcttgcttggag |

The nucleotide sequence of cDNA corresponding to the coding region of organellar GK of pea (PsGKpkm) was obtained from an expressed sequence tag database (22). With the use of an RNeasy Mini Kit (Qiagen, Hilden, Germany), total RNA was extracted from leaves of 2-week-old pea (P. sativum cv. Toyonari; Sakata Seed Co., Yokohama, Japan) seedlings that had been grown in a chamber at 25 °C under a daily cycle of 16 h of white light and 8 h of darkness. The RNA was subjected to RT-PCR with primers OsGKc-forward-1 and OsGKc-reverse-1. The OSKpkm was amplified from the resulting pea cDNA by two-step PCR with primers PsGKpm-forward-1 and PsGKpm-reverse-1 for the first PCR, and PsGKpm-forward-2 and PsGKpm-reverse-2 for the second PCR. The second PCR product was cloned directly into pTA2, and the NclI-BamHI segment of the resultant plasmid was cloned into the corresponding sites of PET19b.

Expression and Purification of GK Enzymes—All proteins were expressed in E. coli BL21(DE3) and purified with the use of TALON metal affinity resin (BD Bioscience). The E. coli cells harboring expression vectors were grown at 37 °C in LB medium containing either ampicillin (100 μg ml⁻¹) for EcGK, BsGK, OsGKpkm, PsGKc, AtGK3, and SeGK or kanamycin (50 μg ml⁻¹) for ScGK, OsGKpkm, and PsGKpm. After the cultures had achieved an optical density at 600 nm of 0.5, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 100 μM and the cells were incubated for an additional 5 h at 25 °C. The cells were then harvested by centrifugation and disrupted by ultrasonic treatment in a solution containing 50 mM sodium phosphate buffer (pH 7.0), 0.5 M NaCl, 0.1% Triton X-100, and 20% glycerol. The cell extracts were centrifuged at 20,400 g for 15 min at 4 °C, and the resulting supernatants were applied to TALON metal affinity resin. The resin was washed with the cell disruption solution supplemented with 18 mM imidazole, and proteins were then eluted with the same solution containing 180 mM imidazole. The concentration of the purified proteins was determined with the use of protein assay kit (Bio-Rad) with BSA as the standard.

Immunoblot Analysis—Rice (O. sativa L. Japonica Nipponbare) leaves were frozen in liquid nitrogen and disrupted in a solution containing 50 mM Tris-HCl (pH 8.0), 7 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, and 1 mM DTT with the use of a Micro Smash instrument (Takara Bio). The extract was centrifuged at 8000 × g for 10 min at 4 °C, and the resulting supernatant (10 μg of protein) was subjected to SDS-PAGE. The separated proteins were transferred to an Immobilon-P transfer membrane (Merck, Darmstadt, Germany), which was then exposed for 1 h at room temperature to 2% ECL Advance Blocking Agent (GE Healthcare) in a solution containing 150 mM NaCl, 0.1% Tween 20, and 20 mM Tris-HCl (pH 7.5). The membrane was then probed consecutively with rabbit antibodies to OsGKpkm (16) at a dilution of 1:5,000 and HRP-conjugated donkey antibodies to rabbit IgG (GE Healthcare) at a dilution of 1:5,000.
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1:20,000, both of which were diluted with Can Get Signal Immunoreaction Enhancer Solution (Toyobo). Immune complexes were then detected with Lumigen TMA-6 solution (GE Healthcare) and visualized with the use of an LAS-1000 instrument (Fuji Film, Tokyo, Japan).

**GK Assay by Autoradiography**—The GK reaction was initiated by the addition of 10 μl of enzyme to 15 μl of a solution containing 100 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 40 μM ATP, [γ⁻³²P]ATP (10 μCi ml⁻¹) (Muromachi Kagaku Kogyo, Tokyo, Japan), and 50 μM GMP, and with or without ppGpp (TriLink Biotechnologies, San Diego, CA) at the indicated concentrations. The mixture was then incubated at 30 °C for the indicated times before termination of the reaction by the addition of 1 μl of 90% formic acid. After the further addition of 10 μl of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) saturated with deionized water, the mixture was agitated and then centrifuged at 9100 × g for 10 min at 4 °C. A 5-μl portion of the upper layer was spotted onto a polyethyleneimine-cellulose F thin-layer sheet (Merck). Chromatography was performed with a solution prepared by dissolving 74 g of (NH₄)₂SO₄, 0.4 g of (NH₄)HSO₄, and 2 g of EDTA (disodium salt) in 100 ml of deionized water. The chromatogram was exposed to an imaging plate, and the associated radioactivity was detected with the use of a Typhoon FLA 7000 instrument (GE Healthcare).

**GK Assay by Spectrophotometry**—GK reactions were initiated by the addition of 40 μl of purified protein (10 nm for EcGK, PsGKpm, PsGKc, or AtGK3, and 100 nm for BsGK, OsGKpm(ND45), or OsGKc) to 60 μl of a solution containing 100 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 40 μM ATP, 50 μM GMP, 1.5 mM phosphoenolpyruvic acid, 150 μM NADH, and 2.5 μl of a mixture of pyruvate kinase and lactate dehydrogenase from rabbit muscle (Sigma), and with or without ppGpp at the indicated concentrations. The reaction was monitored immediately by measurement of A₃₄₀ with a Bio-Spec-1600 spectrophotometer (Shimadzu, Kyoto, Japan) to detect the conversion of NADH to NAD⁺ (14, 23). The initial velocity of the reaction (v₀) was determined as the slope of the A₃₄₀ Curve within the linear portion.

**Assay of GK Activity in Chloroplast Extracts**—Isolation of intact chloroplasts from pea (P. sativum cv. Toyonari) and preparation of a chloroplast extract were performed as previously described (21), with minor modifications. All procedures were performed at 4 °C in a dark room unless indicated otherwise. Intact chloroplasts were isolated from 2-week-old pea seedlings that had been grown in a chamber at 25 °C under a daily cycle of 16 h of white light and 8 h of darkness. Frozen chloroplasts (460 mg) were thawed on ice, suspended in 184 ml of a solution containing 100 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 1 mM DTT, and disrupted by repeated passage through a syringe (20 strokes). The lysate was centrifuged at 20,400 × g for 10 min, and the resulting supernatant was further centrifuged at 30,000 × g for 30 min. The new supernatant was subjected to gel filtration on a Sephadex G-25 column (GE Healthcare) that had been equilibrated with a solution containing 100 mM Tris-HCl (pH 7.5), 100 mM KCl, and 10 mM MgCl₂, and the flow-through fraction (chloroplast extract) was collected and stored at −80 °C until assay. The GK reaction was initiated by the addition of 3 μl of chloroplast extract (15 A₂₆₀ units ml⁻¹) to 12 μl of a solution containing 100 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 50 μM ATP, 50 μM GMP, and [α⁻³²P]GTP (10 μCi ml⁻¹) as well as either with or without ppGpp. After incubation at 30 °C for the indicated times, the reaction was terminated by the addition of 1 μl of 90% formic acid. After the further addition of 10 μl of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) saturated with deionized water, the mixture was agitated and then centrifuged at 9100 × g for 10 min at 4 °C. A 2-μl portion of the upper layer was then spotted onto a polyethyleneimine-cellulose F thin-layer sheet (Merck). One-dimensional TLC was performed with solvent Tb (0.75 M Tris, 0.45 M HCl, 0.5 M LiCl), and two-dimensional TLC was performed with solvent Tb and solvent Sb (prepared by dissolving 74 g of (NH₄)₂SO₄, 0.4 g of (NH₄)HSO₄, and 4 g of EDTA (disodium salt) in 100 ml of deionized water) for the first and second dimensions, respectively. Each chromatogram was then exposed to an imaging plate, and the associated radioactivity was detected with a Typhoon FLA 7000 (GE Healthcare). The [α⁻³²P]GMP for the GK reaction was prepared by treatment of [α⁻³²P]GTP (Muromachi Kagaku Kogyo) with snake venom phosphodiesterase I (Worthington, Lakewood, NJ) for 4 h at 37 °C in a solution containing 20 mM Tris-HCl (pH 7.5) and 100 μM MgCl₂. The reaction mixture was then incubated at 95 °C for 10 min to denature the enzyme before centrifugation at 20,400 × g for 20 min at 4 °C. The resulting supernatant was collected and used for the GK assay after confirmation of the complete conversion of [α⁻³²P]GTP to [α⁻³²P]GMP by TLC.

**Determination of Nucleotide Profiles of E. coli**—The E. coli wild-type strain W3110 was grown at 37 °C in MOPS medium (40 mM MOPS and 4 mM Tricine (adjusted to pH 7.2 with KOH), 50 mM KCl, 10 mM NH₄Cl, 0.5 mM MgSO₄, 0.2 mM KH₂PO₄, 10 mM FeCl₃) supplemented with 0.4% glucose and 0.2% casamino acids. After the culture had achieved an optical density at 600 nm of 0.4, serine hydroxamate was added to a final concentration of 1 mg ml⁻¹ and the cells were incubated at 37 °C for an additional 10 min. The cells (∼1.5 g wet weight) were then harvested by centrifugation and disrupted by ultrasonic treatment in 30 ml of 100% methanol. The cell extract was centrifuged at 9100 × g for 10 min at 4 °C, and the resulting supernatant was transferred to a separation funnel and mixed with 50 ml of chloroform and 20 ml of water. The aqueous phase was collected and dried in an evaporator, and the dried material was dissolved in a low-ionic strength buffer (7 mM KH₂PO₄ adjusted to pH 4.0 with H₃PO₄). The resulting solution was passed through a Cosmosanic filter W (diameter of 13 mm, pore size of 0.45 μm; Nacalai Tesque, Kyoto, Japan) to remove debris and then applied to a Partisil SAX-10 column (GL Science, Tokyo, Japan) for HPLC analysis with an L-2000 series system (Hitachi, Tokyo, Japan). The low-ionic strength buffer was applied to the column at a flow rate of 1 ml min⁻¹, and elution was then performed by increasing the ratio of a high-ionic strength buffer (0.5 M KH₂PO₄ and 0.5 M Na₂SO₄, adjusted to pH 5.4 with KOH) from 0 to 100% over 20 min. The latter buffer was then maintained for 40 min. The amount of nucleotides was determined as peak area by analysis software (EZChrom Elite for Hitachi, Hitachi).
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Expression and Characterization of GK Proteins from Rice—Plants harbor two distinct types of GK, the cytosolic GKc and organellar GKPm (15, 16). To investigate the enzymatic functions of these two proteins of rice, OsGKc and OsGKpm, we expressed them in and purified them from E. coli. We first examined the ND92 form of OsGKpm (Fig. 2A), which was described previously (16), and we found that its specific activity was low compared with that of other GK enzymes. Given that a signal peptide of OsGKpm was based on sequence alignment, it was possible that the ND92 form might not correspond to the actual mature portion of the enzyme. To examine this possibility, we designed, produced, and purified three NH2-terminal truncated OsGKpm derivatives (ND92, ND73, and ND45) with the same NH2-terminal His6 tag sequence (Fig. 2, A and B). Immunoblot analysis of a rice leaf extract revealed that the molecular size of the endogenous OsGKpm protein was ~26 kDa (Fig. 2C), which, after taking into account the His6 tag sequence, was most closely matched by OsGKpm(ND45). An autoradiography-based assay of GK catalytic activity also revealed that, whereas all three recombinant OsGKpm proteins possessed catalytic activity, OsGKpm(ND45) exhibited the highest specific activity (Fig. 2D). We therefore selected OsGKpm(ND45) as being the most representative of endogenous OsGKpm for subsequent biochemical characterization. For comparison, we also cloned, expressed, and purified GK proteins of B. subtilis (BsGK), S. cerevisiae (ScGK) (24), and E. coli (EcGK) (25).

We expressed and purified the recombinant GK proteins OsGKpm, OsGKc, ScGK, BsGK, and EcGK (Fig. 3A). All of the purified GK proteins exhibited the expected catalytic activity, the conversion of GMP to GDP with ATP as the phosphate donor (Fig. 3B). We next examined the effects of ppGpp on each GK protein by supplementation of the reaction mixture with this nucleotide. The catalytic activity of OsGKpm, like that of BsGK, was found to be sensitive to ppGpp (Fig. 3, C-E). In contrast, the activities of OsGKc and ScGK, both of which are cytosolic enzymes, were largely unaffected by ppGpp (Fig. 3C). Furthermore, the activity of EcGK was not inhibited by ppGpp even at a concentration of 200 μM (Fig. 3C), indicating that the E. coli and B. subtilis enzymes differ markedly in their responsiveness to ppGpp.

To compare catalytic function among the GK proteins, we next determined kinetic parameters with the use of a spectrophotometric assay coupled to pyruvate kinase and lactate dehydrogenase (23). The high reproducibility of this assay system allowed kinetic parameters to be calculated from Lineweaver-Burk plots (Fig. 4). The estimated specific activities (units mg−1 protein) were 52 for OsGKpm, 99 for OsGKc, 531 for EcGK, and 133 for BsGK. The kinetic analysis revealed that inhibition constants ($K_i$) for ppGpp relative to the substrate GMP were 2.8 μM for OsGKpm and 13 μM for BsGK (Table 3). The $K_i$ values...
for GMP were 73 μM for OsGKpm and 165 μM for BsGK (Table 3). We also confirmed that GTP and GDP did not affect the catalytic activities of BsGK and OsGKpm (Fig. 3D), indicating the absence of feedback regulation by these products. The IC_{50} of ppGpp for OsGKpm was estimated at 10 μM under the chosen assay conditions (Fig. 5A), which were identical to those previously adopted for BsGK (14). These results thus revealed that ppGpp at low concentrations specifically inhibits the activities of OsGKpm and BsGK. Furthermore, the sensitivity of OsGKpm to ppGpp appeared to be greater than that of BsGK (IC_{50} ~ 30 μM) (14). The kinetic analysis also confirmed that the catalytic activities of OsGKc and EcGK are insensitive to ppGpp (Fig. 4, Table 3).

GKpm Activity of Pea Chloroplast Extracts Is Inhibited by ppGpp in Vitro—To examine endogenous GKpm activity and its regulation by ppGpp, we prepared extracts from isolated pea chloroplasts and measured GK activity with the autoradiography-based assay. The extracts were incubated with [γ-32P]ATP and the nucleotides were analyzed by one- or two-dimensional TLC followed by autoradiography. The generation of both [γ-32P]GDP and [γ-32P]GTP from [γ-32P]GMP was observed in the absence of ppGpp, whereas the production of both of these labeled nucleotides was markedly inhibited in the presence of 50 μM ppGpp (Fig. 6).

To determine directly the ppGpp sensitivity of pea GK enzymes, we isolated pea cDNAs encoding organellar GK (PsGKpm) and cytosolic GK (PsGKc) and prepared the corresponding recombinant proteins (Fig. 7A). We also prepared Arabidopsis organellar GK (AtGK3) protein and examined its enzymatic function together with that of the pea GK proteins. Both organellar GKS, PsGKpm and AtGK3, manifested ppGpp-sensitive activity, whereas the cytosolic enzyme, PsGKc,
showed ppGpp-insensitive activity (Fig. 7B). These results thus demonstrated that both of these dicotyledonous plants also possess a ppGpp-sensitive organellar GK enzyme. The IC$_{50}$ values of ppGpp for PsGKpm and AtGK3 were found to be $\sim$30 and $\sim$50 $\mu$M, respectively, indicating that the level of ppGpp sensitivity of organellar GKS varies among vascular plants.

**Guanylate Kinase of S. elongatus Is a ppGpp-insensitive Enzyme**—Chloroplasts originate from the endosymbiosis of cyanobacteria-like species. To test whether, like plant chloroplasts, cyanobacteria also possess a ppGpp-sensitive GK enzyme, we cloned, expressed, and characterized GK from *S. elongatus* PCC 7942 (Fig. 7A). The catalytic activity of *S. elongatus* GK (SeGK) was not affected by ppGpp at concentrations up to 200 $\mu$M in vitro (Fig. 7, B and C), revealing that cyanobacterial GK, like the *E. coli* enzyme, is insensitive to ppGpp. This result thus indicates that SeGK and GKpm have undergone divergent evolution with regard to regulation by ppGpp.

**Accumulation of ppGpp Has Little Effect on the Guanine Nucleotide Profile of E. coli**—The cellular level of GTP in *B. subtilis* is regulated by the accumulation of ppGpp as a result of the ppGpp-sensitive activity of GK (14, 27). In contrast to the *B. subtilis* enzyme, we found that the activity of *E. coli* GK is insensitive to ppGpp (Fig. 4, Table 3). To examine the effect of ppGpp on the cellular GTP level in *E. coli*, we determined the nucleotide profiles of this bacterium before and after induction of the stringent response by the addition of serine hydroxamate to the culture medium and consequent nutrient shift-down. HPLC analysis revealed that the levels of GTP and GDP were only slightly reduced in cells treated with serine hydroxamate compared with those in the control cells (Fig. 8). The consumption of GTP (or GDP) for ppGpp synthesis may thus reduce the amounts of GTP and GDP, but, in contrast to the pronounced accumulation of ppGpp, the decline in the levels of GTP and GDP seems modest. On the other hand, the cellular level of ATP appeared to be little affected by the accumulation of ppGpp in *E. coli*.

**DISCUSSION**

Our results indicate that the activity of chloroplast GKS of rice, pea, and *Arabidopsis* is sensitive to ppGpp. As far as we are...
aware, this is the first demonstration that the activity of a GK of plant chloroplasts is regulated by ppGpp. Of note, the affinity of OsGKpm for ppGpp ($K_i/H_{11005}$ $2.8 \mu M$) was found to be markedly higher than that for the substrate GMP ($K_m/H_{11005}$ $73 \mu M$). In contrast to chloroplast GKs, we found that the activity of cytosolic GKs of rice and pea is completely resistant to ppGpp. Given that ppGpp biosynthetic enzymes are localized to chloroplasts (28), it is reasonable to conclude that chloroplast GK is under the control of ppGpp signaling in vivo. The specific inhibition of OsGKpm by ppGpp is similar to that observed with its B. subtilis counterpart, which showed $K_i^{ppGpp}$ and $K_m^{GMP}$ values of 13 and 165 $\mu M$, respectively. The endogenous GKpm activity in extracts of isolated pea chloroplasts was also shown to be completely inhibited by ppGpp at a concentration of 50 $\mu M$, in good agreement with the estimate of $\sim 30 \mu M$ for the IC$_{50}$ of ppGpp for recombinant PsGKpm in vitro. Our metabolic labeling analysis confirmed that ppGpp attenuated the conversion of GMP to GTP by inhibiting GKpm activity in the chloroplast extracts. In this analysis, in addition to the spots corresponding to GDP and GTP, we observed labeled spots corresponding to ADP and ATP (Fig. 6), which we had also detected in a previous study (21). Given that the initial substrates for this assay were [$\alpha^{-32}P$]GMP and nonlabeled ATP, the generation of labeled adenosine phosphates cannot be explained simply by the action of adenylate kinase and nucleoside diphosphate kinase present in the extracts. Instead, the generation of [$\gamma^{-32}P$]ATP or [$\beta^{-32}P$]ADP was likely due to an intrinsic phosphatase activity that exchanges the $\gamma$-phosphate of ATP or the $\beta$-phosphate of ADP for [$^{32}P$]orthophosphate.
It has been postulated that most of the enzymes necessary for de novo biosynthesis of IMP in plants localize to organelles (Fig. 1) (17). As in other organisms, the AMP and GMP synthetic pathways in plants diverge at IMP. However, in plants, the pathway for the conversion of IMP to GMP appears to operate in the cytosol, whereas that for the conversion of IMP to AMP takes place in organelles. Furthermore, whereas chloroplasts are thought to possess all of the enzymes required for the synthesis of ATP from IMP, the pathway from GMP to GTP appears to operate in the cytosol, whereas for the conversion of IMP to AMP takes place in organelles. Furthermore, whereas chloroplasts are thought to possess all of the enzymes required for the synthesis of ATP from IMP, the pathway from GMP to GTP appears to operate in both chloroplasts and the cytosol (17) (Fig. 1, Table 1). It is likely that the two enzymes, IMP dehydrogenase and GMP synthase, which together catalyze the conversion of IMP to GMP, do not function in chloroplasts. With regard to the mechanism by which IMP is supplied to the cytosolic de novo GMP pathway in the cytosol, plant plastids possess an adenine nucleotide uniporter, designated BT1, which exports AMP, ADP, and ATP from the stroma to the cytosol (29) and plants convert AMP to IMP through the action of AMP deaminase in the cytosol (30). The export of AMP from chloroplasts via BT1 and the subsequent conversion of AMP to IMP by AMP deaminase may thus provide a source of IMP in the cytosol (17). However, inhibition of AMP deaminase activity was found to barely affect the GTP level in plant cells (31), indicating that AMP deaminase is not likely responsible for providing the major source of IMP for GTP synthesis in the cytosol of plants. Although chloroplast transporters for IMP and GMP remain elusive, IMP produced in chloroplasts might be exported to the cytosol, and GMP formed from IMP in the cytosol might then be imported back into chloroplasts (Fig. 1). The cytosolic production of GMP and the putative GMP transport system may thus determine the flow of GMP to chloroplasts. This partial disjunction of the GMP pathway in plants also renders GKpm responsible for the first step in the conversion of GMP to GTP in chloroplasts and renders the cytosolic GTP pathway independent from guanine ribonucleotide metabolism in chloroplasts. We suggest that plant cells have evolved this specific regulatory system, which depends on the ppGpp sensitivity of GKpm, to control the size of the GTP pool in chloroplasts. The identification of transporters responsible

FIGURE 7. Activity assays for recombinant GK proteins of pea, Arabidopsis, and S. elongatus PCC 7942. A, SDS-PAGE analysis of purified recombinant P. sativum organellar GK (PsGKpm) (2.5 μg), P. sativum cytosolic GK (PsGKc) (1 μg), A. thaliana organellar GK (AtGK3) (2.5 μg), and S. elongatus GK (SeGK) (2.5 μg) proteins. The gel was stained with Coomassie Brilliant Blue, and the arrowheads indicate the purified proteins. B, plots of relative activity versus ppGpp concentration for the conversion of GMP (50 μM) to GDP by purified PsGKpm, PsGKc, AtGK3, or SeGK (each at 10 nM). Enzyme activity was measured as described in the legend to Fig. 4. Data are mean ± S.D. for three separate reactions performed with the same preparation of each purified protein. C, assay of purified SeGK (10 nM) for GK activity with GMP and [γ-32P]ATP as substrates. The assay was performed for the indicated times in the absence of ppGpp (left) or for 3 min in the presence of the indicated concentrations of ppGpp (right). Data are representative of three separate reactions performed with the same preparation of purified protein.
Diversity in ppGpp Sensitivity among Guanylate Kinases

The cellular concentration of ppGpp in *B. subtilis* under the stringent condition of lysine starvation was found to increase to ~67 µM, whereas the GTP level was found to decline by 83%, from 1.37 to 0.23 mM (27). Our kinetic analysis thus confirmed that ppGpp inhibition of BsGK in vitro occurs within the physiological range of ppGpp concentrations. The accumulation of ppGpp achieves even higher levels (~1 mM) in *E. coli* exposed to stringent conditions and again was previously associated with a pronounced reduction in the level of GTP (32). In contrast, we found that the GTP level in *E. coli* was largely unaffected by ppGpp accumulation during a stringent response, even though the ppGpp peak under the stringent condition was markedly larger than the GTP peak (Fig. 8). This finding reflects the ppGpp insensitivity of GTP biosynthesis in *E. coli* and is consistent with our demonstration that the catalytic activity of EcGK is not affected by ppGpp. The function of GK with regard to ppGpp sensitivity thus differs among bacterial species.

The stringent response results in distinct phenotypes in the two model bacteria *B. subtilis* and *E. coli* (14). Extensive molecular biological studies have indicated that the primary target of ppGpp in *E. coli* is RNA polymerase and that ppGpp cooperates with the transcription factor DksA in transcriptional regulation (33, 34). The transcriptional system of *E. coli* has thus been found to be inhibited by ppGpp with an IC₅₀ of ~12 µM (34), whereas RNA polymerase of *B. subtilis* is insensitive to ppGpp (34). The vital role of ppGpp in *B. subtilis* appears to be control of the cellular GTP level (14). The decline in the GTP level in *B. subtilis* during the stringent response results in inhibition of the transcription of specific genes for which the nucleotide required for transcriptional initiation is GTP (26). Replacement of guanine as the base of the transcriptional initiation nucleotide (position +1) with adenine prevented the attenuation of transcription at a gene known to be negatively regulated during the stringent response (35, 36). Thus, although the mechanism differs from that in *E. coli*, the accumulation of ppGpp in *B. subtilis* also eventually results in changes in the transcription of specific genes. In the present study, we confirmed that the cellular levels of GTP and GDP in *E. coli* remain essentially unchanged after ppGpp accumulation (Fig. 8). Thus, as previously proposed (26), there appear to be at least two distinct mechanisms of transcriptional regulation by ppGpp in the bacterial kingdom, and that the difference between these mechanisms likely reflects distinct targets of ppGpp, namely RNA polymerase on the one hand and an enzyme of the GTP biosynthetic pathway on the other. Our data now indicate that plant chloroplasts possess a *B. subtilis*-type transcriptional regulatory system. Analysis of the transcription of genes in the chloroplast genome should provide insight into the downstream events triggered by ppGpp inhibition of GKpm.

We also found that the cyanobacterium *S. elongatus* PCC 7942 possesses a ppGpp-insensitive GK. Although whether other species of cyanobacteria will also be found to express a similar GK enzyme remains to be determined, our observations suggest that GKS of plant chloroplasts have acquired ppGpp sensitivity during plant evolution. We were not able to identify a signature of ppGpp sensitivity by comparing the amino acid sequences (with ClustalW) or structures of bacterial GK proteins. Further studies, especially structural analysis of the ligand

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**FIGURE 8. Nucleotide profiles of *E. coli* under control and stringent conditions.** Cultures of *E. coli* were incubated without (middle panel) or with (bottom panel) serine hydroxamate (SHX, 1 mg ml⁻¹) for 10 min, after which nucleotide profiles of the cells were determined by HPLC. The HPLC profiles obtained with 5 nmol of standard nucleotides (GMP, GDP, ATP, GTP, or ppGpp) are also shown (top panel).
binding state of specific GK proteins, should help to clarify the mechanism of inhibition of GK activity by ppGpp in particular species. Such studies might also provide a basis for the development of new antibacterial strategies for the treatment of infectious diseases.

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