DOI: 10.1002/cbic.200800737

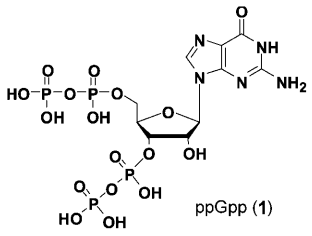
Bacterial Alarmone, Guanosine 5′-Diphosphate 3′-Diphosphate (ppGpp), Predominantly Binds the β′ Subunit of Plastid-Encoded Plastid RNA Polymerase in Chloroplasts

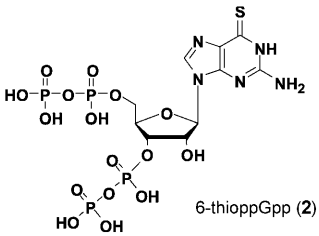
Michio Sato,[a] Kosaku Takahashi,*[a] Yuka Ochiai,[a] Takeshi Hosaka,[b, c] Kozo Ochi,[b] and Kensuke Nabeta[a]

Chloroplasts, which are thought to have originated from cyanobacteria, have their own genetic system that is similar to that of the bacteria from which they were derived. Recently, bacterial alarmone guanosine 5′-diphosphate 3′-diphosphate (ppGpp, **1**), a key regulatory molecule that controls the stringent response, was identified in the chloroplasts of plant cells. Similar to its function in bacteria, ppGpp inhibits chloroplast RNA polymerase; this suggests that ppGpp mediates gene expression through the stringent response in chloroplasts. However, a detailed mechanism of ppGpp action in chloroplasts remains elusive. We synthesized 6-thioguanosine 5′-diphosphate 3′-diphosphate (6-thioppGpp) as a photoaffinity probe of ppGpp; this probe thus enabled the investigation of ppGpp binding to chloroplast RNA polymerase. We found that 6-thioppGpp, as well as ppGpp, inhibits chloroplast RNA synthesis in vitro in a dose-dependent manner. Cross-linking experiments with 6-thioppGpp and chloroplast RNA polymerase indicated that ppGpp binds the β′ subunit (corresponding to the *Escherichia coli* β′ subunit) of plastid-encoded plastid RNA polymerase composed of α, β, β′, β″, and σ subunits. Furthermore, ppGpp did not inhibit transcription in plastid nucleoids prepared from tobacco BY-2 cells; this suggests that ppGpp does not inhibit nuclear-encoded plastid RNA polymerase.

Introduction

Among bacteria, one of the most significant systems that regulates gene expression is the stringent response, which enables cells to adapt to stressful conditions such as nutrient deprivation. Guanosine 5′-diphosphate 3′-diphosphate (ppGpp) plays an important role as a key mediator of the stringent response


ppGpp (**1**)


6-thioppGpp (**2**)

and is thus called bacterial alarmone.[1] In bacteria, ribosome-dependent ppGpp synthesis is catalyzed by the *relA* gene product, ppGpp synthetase, which is expressed in response to the binding of uncharged tRNA to the ribosomal A site. The binding of ppGpp to RNA polymerase (RNAP) causes either activation or suppression of various genes. The stringent response is involved in the induction of secondary metabolism and differentiation in bacteria.[2,3] The significance of ppGpp in bacterial physiology has drastically increased with the recent discovery that ppGpp induces bacterial quorum sensing, which consists of a range of intercellular signalling mechanisms to determine population density[4,5] and controls various aspects of pathogenic and symbiotic bacterium–host interactions.[6–9]

In plants, chloroplasts have their own genomes and mechanisms for gene expression.[10] In addition to photosynthesis, there are many similarities between chloroplasts and cyanobacteria. With only a few exceptions, phylogenetic trees based on proteins reveal that all chloroplasts in plants and cyanobacteria are congenetic; this strongly supports the idea that chloroplasts originated from cyanobacteria.[11] RNAPs in plastids are classified into two categories: one is plastid-encoded plastid RNA polymerase (PEP) and the other is nuclear-encoded plastid RNA polymerase (NEP).[12,13] PEP, which is similar to bacterial RNAP, contains a core complex composed of α, β, β′, and β″ subunits and a number of accessory proteins.[14–16] NEP is analogous to T7 phage RNAP, and at least two kinds of NEP (RpoTp and RpoTmp) exist in plastids. RpoTp is imported to plastids, and RpoTmp is transferred to both plastids and mitochondria.[17,18] In maize and mustard (*Sinapis alba* L.), it was reported that the bacterial property of PEP appears in etioplasts and greening chloroplasts.[19,20] Differential control of NEP and

[a] *M. Sato, Dr. K. Takahashi, Y. Ochiai, Prof. K. Nabeta*
Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University
Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589 (Japan)
Fax: (+81) 11-706-2505
E-mail: kosaku@chem.agr.hokudai.ac.jp

[b] *Dr. T. Hosaka, Dr. K. Ochi*
National Food Research Institute
2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642 (Japan)

[c] *Dr. T. Hosaka*
Present address: Fiber-Nanotech Young Researcher Empowerment Center Shinshu University, 8304 Minamiminowa, Nagano 399-4598 (Japan)

PEP activities causes dynamic changes in plastid gene expression during plastid differentiation. In the process of evolution, plants seem to specialize their cellular functions, and this eventually leads to the ability to differentiate plastids variedly.

Despite the fact that the genetic systems between chloroplasts and bacteria are homologous, the functional capability of ppGpp in plant cells has been largely overlooked. Recently, *relA/spoT* homologous genes (*rsh*) encoding a protein with ppGpp synthetic activity were found in several plants.[21–24] Most plant RSH proteins involve chloroplast transit sequences in their N-terminal regions. These results harmonize with analytical data showing that ppGpp localizes in chloroplasts.[25] Furthermore, ppGpp inhibits chloroplast RNAP activity dose-dependently in vitro. Judging from these previous works, it seems highly likely that the bacterial-type stringent response regulated by ppGpp also exists in higher plants. Supporting this notion is the fact that ppGpp production in plants is induced under stressful conditions, most likely mediated by plant hormones.[25] Furthermore, in addition to the *rsh* genes, novel *relA/spoT* homologues known as *crsh* (Ca^{2+}-activated *relA/spoT* homologue), which contain two EF-hand motifs at the C terminus, were reported in rice and *Arabidopsis thaliana*.[26,27] Moreover, *crsh* in *A. thaliana* was shown to play a role in plant fertilization.[27] These investigations are quite intriguing from the view of stress adaptation, plant fertilization and the evolution of plants and chloroplasts. However, the details of ppGpp function in plants remain elusive.

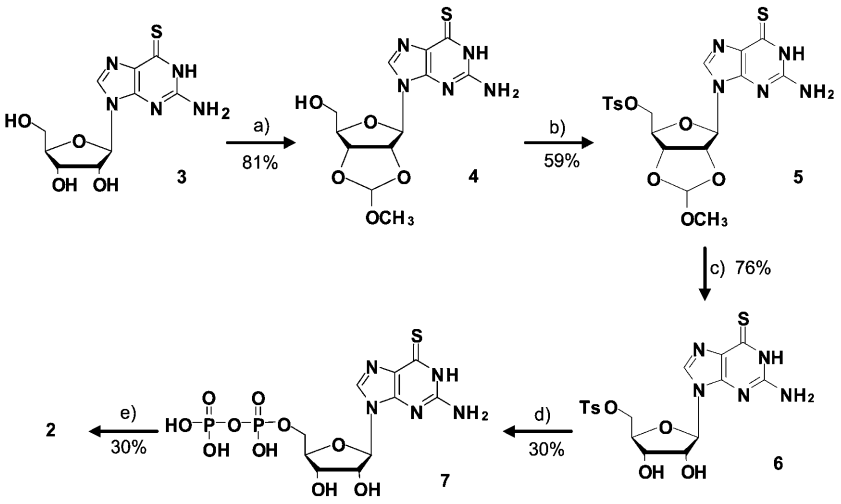
To identify the ppGpp-binding subunit in *E. coli* RNAP, radioactive cross-linkers 8-N_3ppGpp[28] and 6-thioppGpp (**2**)[29] have been used. Binding experiments with these compounds however have given rise to different results, in which 8-N_3ppGpp and 6-thioppGpp differentially bind to the β and β′ subunits, respectively. The position of the cross-linking moiety on guanine appears to render these cross-linkers different in their binding to *E. coli* RNAP subunits. The recent X-ray crystal structure of *Thermus thermophilus* RNAP complexed with ppGpp demonstrates that ppGpp binds proximally to the active site located between β and β′ subunits, and that the oxygen of the carbonyl group at the 6-position of ppGpp is vicinally oriented to the β′-subunit in RNAP.[30] As a first step in clarifying the biochemical activity of ppGpp on chloroplast RNAP, we synthesized 6-thioppGpp by using chemical and enzymatic reactions and identified the subunit to which ppGpp binds in chloroplast RNAP. The present data also suggest that ppGpp plays an important role in the regulation of chloroplast gene transcription.

Results and Discussion

Synthesis of 6-thioppGpp

Previously, radioactive 6-thioppGpp was prepared from 6-thioguanosine 5′-triphosphate (6-thioGTP) and [γ-^{32}P]ATP by the phosphate transfer reaction of crude RelA enzyme obtained from *E. coli*.[29] Because 6-thioGTP was the starting material, radioactive 6-thioppGpp was obtained along with radioactive 6-thioguanosine 5′-triphosphate 3′-diphophate (6-thiopppGpp), and hence radioactive 6-thioppGpp needed to be purified by using anion exchange column chromatography. We attempted to synthesize 6-thioppGpp through 6-thioGDP (**7**) to avoid contamination of 6-thiopppGpp with 6-thioppGpp and simplify the separation of radioactive compounds (Scheme 1). The starting material, 6-thioguanosine (**3**), was protected as a 2′,3′-*O*-methoxymethylidene derivative (**4**). The tosyl group was introduced at the 5′-position of **2** (without the other protection group) to give **5** in a reasonable yield (59%) after treatment with *n*BuLi. After deprotection of the acetal group at the 2′- and 3′-positions of **5**, the resulting compound **6** was reacted with tris(tetra-*n*-butylammonium) pyrophosphate to give 6-thioGDP (**7**). Finally, **7** was subjected to an enzymatic reaction of pyrophosphate transferase with ATP to obtain 6-thioppGpp (**2**) as a lithium salt.[30] The completion of this reaction was monitored by HPLC analysis. To synthesize radioactive 6-thioppGpp, 6-thioGDP (**7**) was incubated in a reaction mixture containing [γ-^{32}P]ATP and a pyrophosphate transferase. After 2 h, this reaction mixture was extracted by phenol/chloroform, and to remove traces of phenol, an equal volume of chloroform was added to the aqueous layer. The aqueous solution containing radioactive 6-thioppGpp was used for cross-linking.

Previously, 6-thioGDP was synthesized together with 6-thioGTP; however, the compounds were not easily separated by anion exchange column chromatography.[31] We report here new a synthetic method for 6-thioGDP, an intermediate of 6-



Scheme 1. Synthesis of 6-thioppGpp (**2**). a) $(CH_3O)_3CH$, pyridine-HCl, DMSO, RT, 40 h; b) 1: *n*BuLi, THF, −78 °C, 15 min, 2: tosyl chloride, THF; 0 °C, 1 h; c) TFA, MeOH/H_2O (2:1), RT, 16 h; d) $(Bu_4N)_3HOPP$, CH_3CN, RT, 96 h ; e) ATP, pyrophosphate transferase, glycine buffer (pH 10), 37 °C, 2 h.

thioppGpp, which is easy to carry out and chemically well-defined. These reactions obtained milligram quantities of the final product sufficient for small-scale biochemical experiments. Moreover this synthesis provided 6-thioppGpp without contamination of 6-thiopppGpp. This synthetic method will be able to produce other photoaffinity probes containing GDP as a partial structure.

The inhibitory activity of 6-thioppGpp in chloroplast RNA polymerase reaction

Chloroplasts of spinach (*Spinacia oleracea*) have been used as a model for studying the function of chloroplasts. The complete nucleotide sequence of the plastid chromosome of spinach has been reported.[32] Therefore, we selected spinach chloroplasts for RNAP preparation. Since purification of chloroplast RNAP from mature plant leaves is difficult due to the association of many proteins with chloroplast RNAP,[15] we used partially purified chloroplast RNAP (see the Experimental Section) to examine the inhibitory activities of 6-thioppGpp and ppGpp. As expected, ppGpp and 6-thioppGpp both inhibited the activity of RNAP dose dependently at concentrations ranging from 200 to 1,000 μM (Figure 1). High concentrations of ppGpp were required to inhibit chloroplast RNAP activity substantially. In this regard, it is noteworthy that the inhibitory effect of ppGpp observed in vitro was always much smaller than that observed in vivo.[33,34] Gourse and colleagues[35] suggested that this discrepancy reflects, at least in *E. coli*, the level of the DksA protein, which amplifies the effects of ppGpp. A similar mechanism for amplifying the effect of ppGpp might operate in chloroplasts, though a DksA homologue has not yet been found in plants.

The extent of inhibition by 6-thioppGpp in the RNAP reaction was almost the same as that of ppGpp; this reflects the fact that structural differences between these compounds are minimal. Given that they have the same inhibitory activity and only slight structural differences, 6-thioppGpp and ppGpp are considered to bind the same site on chloroplast RNAP. Thus, we employed 6-thioppGpp for identification of the ppGpp binding site in chloroplast RNAP.

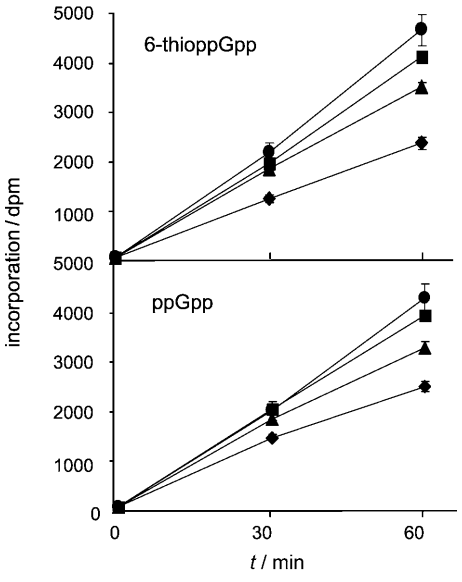


Figure 1. In vitro inhibition of chloroplast RNAP by 6-thioppGpp and ppGpp. Partially purified chloroplast RNAP from spinach (*S. oleracea*) was incubated in the presence of [^{3}H]UTP and various concentrations of 6-thioppGpp and ppGpp. [^{3}H]UTP incorporation was determined by liquid scintillation counting. The mean values from three independent experiments are shown. Vertical bars represent standard errors. ●, no ppGpp and 6-thioppGpp; ■, 200 μM; ▲, 500 μM; ◆, 1000 μM.

Cross-linking of 6-thioppGpp to chloroplast RNA polymerase

In this study, radioactive 6-thioppGpp was converted from 6-thioGDP by pyrophosphate transferase.[30] An equivalent amount of [γ-^{32}P]ATP to 6-thioGDP was added to the reaction mixture. Accordingly, the radioactive nucleotide in the reaction mixture was almost solely radioactive 6-thioppGpp due to effective enzymatic catalysis. The small amount of unreacted [γ-^{32}P]ATP was considered not to pose a serious problem for cross-linking. Therefore, the reaction mixture was not purified. A mixture of radioactive 6-thioppGpp and chloroplast RNAP was subjected to UV irradiation for photoactivation. The results of SDS-PAGE analysis indicate that a protein with a molecular size of 75 kDa cross-linked with radioactive 6-thioppGpp (Figure 2 A). The competition experiments demonstrate that cross-linking of radioactive 6-thioppGpp was severely inhibited by the co-existence of ppGpp (Figure 2 B); this indicates that the binding site of radioactive 6-thioppGpp was identical with that of ppGpp. A broad protein band with the molecular weight of about 66 kDa was identified as bovine serum albumin (BSA), which was contained in cross-linking reaction buffer.

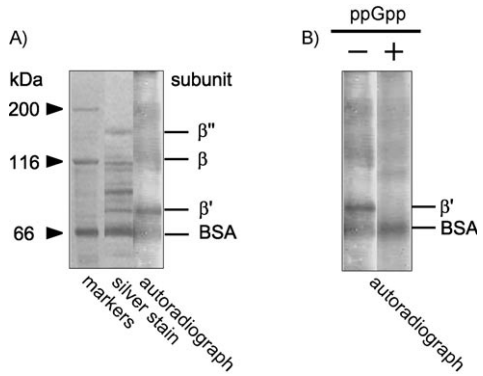


Figure 2. Cross-linking of chloroplast RNAP with radioactive 6-thioppGpp. A) SDS-PAGE of chloroplast RNAP visualized by silver stain and cross-linked with radioactive 6-thioppGpp. B) competition experiment. 100-fold amount of normal ppGpp was added.

Peptide fingerprinting and identification

The protein band corresponding to the radioactive band was excised and digested by trypsin and then its MALDI-TOF MS spectrum was measured. A database search (MASCOT) of

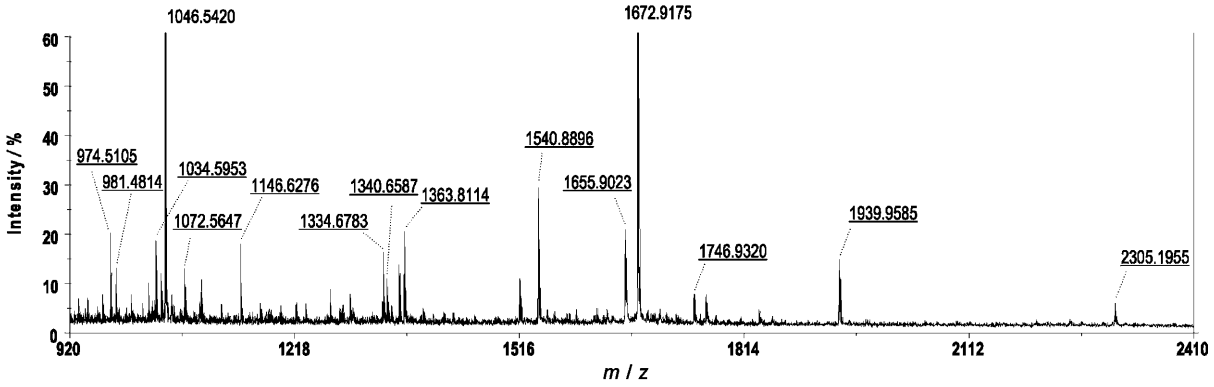


Figure 3. MALDI-TOF/MS spectral data of tryptic digested peptides from the protein cross-linked with radioactive 6-thioppGpp. Both peaks of 1045 and 1672 are angiotensin and neurotensin peptides for internal standard.

major peaks indicated that the protein cross-linked with 6-thioppGpp was the β′ subunit of plastid-encoded plastid RNAP (PEP; Figure 3 and Table 1). Accordingly, we conclude that ppGpp binds the β′ subunit of PEP to inhibit transcription.

Table 1. Peptide mass fingerprinting analysis of the protein corresponding to the radioactive band.

Found (*m/z*)	Calculated (*m/z*)	Amino acid sequence assigned
2305.1955	2305.2226	^{27}ILPNGEIVGEVTKPYTFHYK^{46}
1939.9585	1939.9588	^{173}YSIPLFFTTQGFDTFR^{188}
1746.9320	1746.9683	^{440}ILQEVMQGHPVLLNR^{454}
1655.9023	1655.8962	^{327}LVQEAVDTLLDNGIR^{341}
1540.8896	1540.8845	^{461}LGIQAFQPILVEGR^{474}
1363.8114	1363.8056	^{382}SVIVVGPSLSLHR^{394}
1340.6587	1340.6515	^{287}LMSSDINELYR^{297}
1334.6783	1334.6910	^{303}NNTLTDLLSTSR^{314}
1146.6276	1146.6266	^{654}TTVGHISLYR^{663}
1072.5647	1072.5633	^{201}EQLADLDLR^{209}
1034.5953	1034.5880	^{646}EIIDIYIR^{653}
981.4814	981.4887	^{355}SFSDVIEGK^{363}
974.5105	974.5265	^{191}EISTGAGAIR^{200}

PEP, which is composed of α, β, β′, β″, and σ subunits, has high similarity to cyanobacterial RNAP.[36,37] The plastid genome encodes the genes of the former four subunits, *rpoA*, *rpoB*, *rpoC1*, and *rpoC2*, whereas the nuclear genome encodes the gene of the σ subunit. The subunit corresponding to the β′ subunit in *E. coli* RNAP is divided into the β′ and β″ subunits (75 kDa and 154 kDa) in PEP as well as RNAP of cyanobacteria. The amino acid sequences of β′ and β″ subunits show homology with the N- and C-terminal regions of *E. coli* RNAP (43% and 27%, respectively).[38] The β′ subunit that is found to bind ppGpp in our study has similarity to the β′ subunit of *E. coli* RNAP. The present result was therefore analogous to the results from ppGpp-*T. thermophilus* RNAP crystal structure analysis[30] and *E. coli* RNAP binding experiments with 6-thioppGpp.[29]

Effects of ppGpp on transcription in isolated chloroplast and proplastid nucleoids

Several reports have suggested that plastids of BY-2 cells, which are derived from *Nicotiana tabacum* L. cv. Bright Yellow-2, have functional and morphological properties similar to undifferentiated proplastids from meristematic cells.[39,40] The NEP, which is similar to T7 phage RNAP, is a predominant transcriptional enzyme and is responsible for activation of plastid-encoded transcriptional machinery in proplastids. Since we found that ppGpp binds the β′ subunit of PEP, we next studied whether or not ppGpp selectively inhibits transcriptional activity of chloroplast nucleoids (from matured tobacco leaves) and proplastid nucleoids (from BY-2 cultured cells). Interestingly, ppGpp differentially inhibited transcription of each plastid nucleoid (Figure 4). While transcription in chloroplast nucleoids

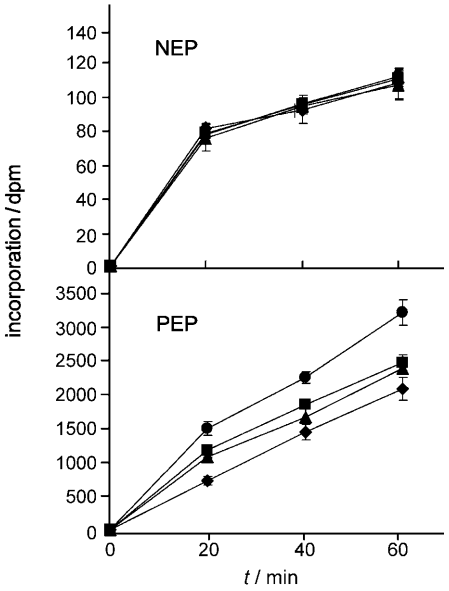


Figure 4. Effects of ppGpp on transcription in isolated chloroplast- (PEP) and plastid- (NEP) nucleoids. The transcription assay was performed in the presence of [^{3}H]UTP and various concentrations of ppGpp. [^{3}H]UTP incorporation was determined by liquid scintillation counting. The mean values from three independent experiments are shown. Vertical bars represent standard errors. ●, no ppGpp; ■, 200 μM; ▲, 500 μM; ◆, 1000 μM.

was evidently inhibited by 200 to 1,000 μM ppGpp, virtually no inhibition was detected in proplastid nucleoids transcription even at 1000 μM ppGpp; this indicates the differential ppGpp sensitivity of each plastid nucleoid. Apparently, this is a reflection of the fact that chloroplast nucleoids and proplastid nucleoids have different transcriptional machinery.[39] Accordingly, we conclude that ppGpp selectively inhibits transcription by PEP (bacterial type RNA polymerase) and exerts its effects after proplastids differentiate into chloroplasts.

NEP initially transcripts a set of genes (rRNA, ribosomal proteins, and PEP) that are involved in the gene expression system in chloroplasts. PEP then carries out transcription of most chloroplast genes. Recently, σ factors and their mode of action have been extensively studied to elucidate the regulatory mechanism of chloroplast transcription.[40] For transcription by PEP, a number of σ factors encoded in nuclei are recruited as the situation demands, and transcription is controlled according to environmental changes and the differentiated state of the plastid.[36,37] Our present data show that inhibitory activity of ppGpp for PEP would be not only under the control of plant hormones such as jasmonic acid,[25] but also depends on the differential stages of plastids.

Conclusions

In the present study, we established an efficient synthetic pathway for 6-thioppGpp, which is a photoaffinity probe of ppGpp that inhibits chloroplast RNAP in vitro as well as ppGpp. The cross-linking of radioactive 6-thioppGpp with chloroplast RNAP indicated that ppGpp binds the β′ subunit of PEP, a bacterial type RNAP in chloroplasts. Furthermore, ppGpp was shown to inhibit PEP functions selectively, but not NEP, which is a phage type RNAP. Thus, ppGpp appears to be an important second messenger that not only regulates chloroplast functions, but also has eventual consequences for the whole plant. The proof that ppGpp acts as an alarmone in plants requires more experimental work, however. The elucidation of the functions of ppGpp would provide new insights into plant physiology, as represented by the gene expression system in chloroplasts.

Experimental Section

Spectrometry: ^{1}H NMR spectra were recorded on Brucker AMX-500 FT NMR (500 MHz) spectrometers, and $CDCl_3$ (7.26 ppm), CD_3OD (3.30 ppm), or [D_6]DMSO (2.49 ppm) were used as internal standards. When measured in D_2O, sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropanoate (0.00 ppm) was employed. ^{31}P NMR spectra were taken on a Brucker AMX-500 FT NMR spectrometer (202 MHz) with phosphoric acid (5%) in D_2O (0.00 ppm) as an external standard. Mass spectra were taken on a Jeol JMS-AX500 mass spectrometer and Voyager MALDI TOF mass spectrometer (Applied Biosystems; Caarlsbad, USA). The specific rotation values were measured on a Jasco DIP-310 instrument. Chemical reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), unless otherwise noted.

2′,3′-*O*-(methoxymethylidene)-6-thioguanosine (4): Trimethyl orthoformate (5 mL, 31.0 mmol) was added by syringe to a flask containing (500 mg, 1.7 mmol) of 6-thioguanosine (**3**, Acros Organics, Geel, Belgium). This was followed by the addition of pyridine hydrochloride (300 mg, 2.6 mmol). DMSO (0.7 mL) was added with stirring and the amber suspension was stirred for 40 h at ambient temperature. The obtained suspension became clear after addition of methanol (10 mL) and sodium methoxide (150 mg, 2.8 mmol), and it was stirred for 3 h. After evaporation of the reaction mixture, the resulting residue was purified by flash column chromatography on silica gel (MeOH/$CHCl_3$ 3:97) to yield **4** as a pale yellow powder (460 mg, 81%). ^{1}H NMR (500 MHz, [D_6]DMSO) diastereoisomers A and B $\delta = 12.0$ (s, 1H; NH), 8.10, 8.07 (s, 1H; H-8), 6.83 (s, 2H; NH_2), 6.12, 6.04 (s, 1H; acetal), 6.06, 5.95 (d, $J = 3.0$ Hz, 1H; H-1′), 5.28 (m, 1H; H-2′), 5.11 (m, 1H; 5′-OH), 5.01 (m, 1H; H-3′), 4.21, 4.12 (q, $J = 3.8$ Hz, 1H; H-4′), 3.54 (m, 2H; H-5′), 3.32, 3.30 (s, 3H; OCH_3); EI-MS (*m/z*) 341 (M^+); HR-MS: calcd for $C_{12}H_{15}N_5O_5S$: 341.0794, found: 341.0758.

2′,3′-*O*-(methoxymethylidene)-5′-*O*-tosyl-6-thioguanosine (5): *n*BuLi (0.16 mL, 1.59 M in *n*-hexane solution) was added to a solution of **4** (40 mg, 0.12 mmol) in dry THF (1 mL) at −78 °C under an argon atmosphere. After stirring for 15 min, the solution was warmed to 0 °C and stirred for additional 10 min at same temperature. A solution of tosyl chloride (25.1 mg, 0.13 mmol) in dry THF (0.5 mL) at 0 °C was then added to the reaction mixture, and stirred at this temperature for 1 h. The reaction mixture was poured into ice-cold water (30 mL) and extracted with CH_2Cl_2 (30 mL). The organic layer was washed with a saturated NaCl aqueous solution (30 mL) and dried over Na_2SO_4. The organic layer was evaporated to afford a residue. The resultant residue was purified by silica gel column chromatography (MeOH/$CHCl_3$ 1.5:98.5) to give **5** (34 mg, 59%): diastereoisomers A and B: ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.69$ (s, 1H; H-8), 7.63 (m, 2H; aromatic-H), 7.20 (m, 2H; aromatic-H), 6.03, 5.90 (d, $J = 2.3$ Hz, 1H; H-1′), 5.98, 5.91 (s, 1H; acetal-H), 5.44, 5.39 (dd, $J = 6.5$, 2.0 Hz, 1H; H-2′), 5.26, 5.15 (dd, $J = 6.5$, 3.0 Hz, 1H; H-3′), 5.22 (s, 2H; NH_2), 4.47 (m, 2H; H-5′), 4.07 (m, 1H; H-4′), 3.39, 3.31 (s, 3H; OCH_3), 2.38 (s, 3H; aromatic-CH_3); FAB-MS (*m/z*): 452 [$M-H$]$^-$; HR-MS: calcd for $C_{19}H_{20}N_5O_7S_2$: 494.0804, found: 494.0816.

5′-*O*-tosyl-6-thioguanosine (6): Trifluoroacetic acid (2.2 mL) was added to a solution of **5** (86 mg, 0.17 mmol) in a mixed solution of MeOH and H_2O (10 mL total, 2:1), and the reaction mixture was stirred at room temperature for 16 h. After concentration in vacuo, the obtained residue was dissolved in a mixed solution of EtOH and H_2O (1:2). The suspension was treated with NH_4HCO_3 (250 mg, 3.2 mmol), and warmed at 40 °C for 15 min. The suspension was then cooled to 4 °C, and was allowed to precipitate at the same temperature for 1 h. After recovery of the precipitate by filtration, it was dried to yield **6** (60 mg, 76%). $[\alpha]_D^{25} = +8.6°$ ($c = 0.13$, DMSO); ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.85$ (s, 1H; H-8), 7.69 (d, $J = 8.1$ Hz, 2H; aromatic-H), 7.27 (d, $J = 8.1$ Hz, 2H; aromatic-H), 5.74 (d, $J = 4.5$ Hz, 1H; H-1′), 4.55 (t, $J = 4.7$ Hz, 1H; H-2′), 4.34 (m, 1H; H-3′), 4.32 (m, 2H; H-5′), 4.14 (m, 1H; H-4′), 2.39 (s, 3H; aromatic-CH_3); FAB-MS (*m/z*): 452 [$M-H$]$^-$; HR-MS: calcd for $C_{17}H_{18}N_5O_6S_2$, 452.0699; found, 452.0697.

6-thioguanosine 5′-diphosphate (6-thioGDP, 7): 5′-*O*-tosylguanosine (**6**, 200 mg, 0.44 mmol) was dissolved in CH_3CN (1 mL), and 795 mg of tris(tetra-*n*-butylammonium) hydrogen pyrophosphate was allowed to react for 96 h at room temperature under an argon atmosphere. To the reaction mixture was added H_2O (10 mL), the resulting suspension was centrifuged, and then the supernatant, diluted with H_2O (60 mL), was loaded onto a DEAE-cellulose column (HCO_3^- form, Whatman, Maidstone, UK). This anion exchange column was washed with NH_4HCO_3 (100 mL of 0.1 M), and eluted with a linear gradient of NH_4HCO_3 (0.1–0.7 M). The fractions containing **7** were combined and lyophilized to obtain **7** (61 mg,

30%). $[\alpha]_D^{25} = -33.1^\circ$ ($c = 0.12$, H_2O); ^1H NMR (500 MHz, D_2O): $\delta =$ 8.25 (s, 1H; H-8), 5.93 (d, $J = 5.9$ Hz, 1H; H-1′), 4.80 (1H; overlapped with solvent peak, H-3′), 4.55 (t, $J = 4.4$ Hz, 1H; H-2′), 4.36 (brs, 1H; H-4′), 4.22 (brt, $J = 4.2$ Hz, 2H; H-5′); ^{31}P NMR (202 MHz, D_2O): $\delta =$ −10.35 (d, $J = 20.0$ Hz), −11.09 (dd, $J = 20.0$, 5.1 Hz); FAB-MS (*m/z*): 460 $[M+H]^+$; HR-MS: calcd for $C_{10}H_{16}N_5O_{10}P_2S$: 460.0093; found: 460.0086.

6-thioguanosine 5′-diphosphate 3′-diphosphate (6-thioppGpp, 2): Synthesis of **2** was performed by using purine nucleotide pyrophosphate transferase from *Streptomyces morookaensis* A-573.[30] The reaction was performed at 37 °C for 1 h in a buffer (10 mL final volume) containing purine nucleotide pyrophosphate transferase (8 units), ATP disodium salt (51.6 mg), **7** (43 mg), $MgCl_2$ (10 mM), and glycine (100 mM, pH 10.0). The reaction mixture was subjected to a DEAE-Sephadex A-25 column (Cl^- form, GE Healthcare) equilibrated with distilled water. The column was eluted with a linear gradient of LiCl (0.1–1.0 M). The fractions containing **2** were collected, filtrated through a Millipore MF membrane (0.22 μm), and concentrated to about 5 mL under reduced pressure. Compound **2** was precipitated by adding EtOH (50 mL), washed twice with EtOH and once with acetone, and lyophilized to remove residual water. Finally, 18.8 mg of **2** was obtained (30%). $[\alpha]_D^{25} = -25.9^\circ$ ($c = 0.37$, H_2O); ^1H NMR (500 MHz, D_2O): $\delta = 8.29$ (s, 1H; H-8), 6.01 (d, $J =$ 6.4 Hz, 1H; H-1′), 5.01 (m, 1H; H-3′), 4.91 (t, $J = 5.9$ Hz, 1H; H-2′), 4.55 (brs, 1H; H-4′), 4.24 (brt, $J = 4.2$ Hz, 2H; H-5′); ^{31}P NMR (202 MHz, D_2O): $\delta = -4.39$ (d, $J = 21.8$ Hz), −5.01 (brs), −8.75 (d, $J = 15.4$ Hz) −9.13 (dd, $J = 21.6$, 8.6 Hz); FAB-MS (*m/z*): 648 $[M-H]^-$; HR-MS: calcd for $C_{10}H_{11}N_5O_{16}P_4SLi_5$: 647.9673, found: 647.9694.

Radioactive 6-thioppGpp: [γ-^{32}P]ATP (250 μCi, MP Biomedicals, Irvine, USA) was diluted with unlabeled ATP (5 mM) and **7** (5 mM) in reaction mixture (30 μL) containing the pyrophosphate transferase. The reaction was carried out at 37 °C for 1 h. The radioactive products thus prepared were stored at −20 °C until use.

Preparation of RNA polymerase from spinach chloroplasts: Mature spinach leaves (*Spinacia oleracea*, 3 kg) were used for isolation of intact chloroplasts.[41] Purification of RNA polymerase was carried out by the method of Briat et al. with some modifications.[42] The isolated chloroplasts were homogenized in a buffer containing Tris-HCl (50 mM, pH 7.8), EDTA (4 mM), $(NH_4)_2SO_4$ (0.1 M), DTT (1 mM), PMSF (0.05 mM). Glycerol was added to the homogenized suspension to a final concentration of 25%, and then the suspension was centrifuged at 80000 *g* for 45 min. The supernatant was loaded onto a Heparin-Sepharose CL-6B (GE Healthcare) column (ϕ1.5×5 cm) equilibrated with the basal buffer (50 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 0.1% Triton X-100, 25% glycerol, 1 mM DTT) containing $(NH_4)_2SO_4$ (0.1 M). The column was washed with the basal buffer (100 mL) containing $(NH_4)_2SO_4$ (0.1 M) and then eluted with a 60 mL linear gradient of $(NH_4)_2SO_4$ (0.1–1.0 M). Fractions (2.0 mL) were collected and a small amount of each fraction was analyzed by transcriptional assay. The RNAP fractions eluted from the Heparin-Sepharose column were combined and dialyzed to equilibrium against the basal buffer containing $(NH_4)_2SO_4$ (15 mM). A solution of Polymin P [0.1% (*v/v*)] was added to the dialyzed solution to a final concentration of 0.002%. After vigorous stirring on ice for 15 min, the solution was centrifuged at 10000 *g* for 30 min. The precipitate was extracted with the basal buffer containing $(NH_4)_2SO_4$ (300 mM) and adjusted to 50 mM $(NH_4)_2SO_4$ by addition of the basal buffer containing $(NH_4)_2SO_4$ (15 mM). The supernatant was applied to a DEAE-cellulose column (DE-52, Whatman, ϕ1.6×4 cm) equilibrated with the basal buffer containing $(NH_4)_2SO_4$ (50 mM). The column was washed with basal buffer (100 mL) containing $(NH_4)_2SO_4$ (50 mM) and then eluted with a 40 mL linear gradient of $(NH_4)_2SO_4$ (50–600 mM). Fractions (2.0 mL each) were collected and used in the transcriptional assay. Following the transcriptional assay, fractions containing RNAP were combined and were precipitated by addition of an equal volume of a saturated $(NH_4)_2SO_4$ solution (pH 8.0). After centrifugation at 10000 *g* for 30 min, the pellets were dissolved in a small volume of the basal buffer containing $(NH4)_2SO_4$ (30 mM) and glycerol (25%), and dialyzed against the same buffer prior to storage at −30 °C. All steps were performed at 4 °C.

Chloroplast RNA polymerase reaction: The enzyme reaction was carried out in a buffer (final volume 20 μL) containing Tris-HCl (50 mM, pH 7.6); $MgCl_2$ (10 mM), KCl (100 mM), ATP (0.2 mM), GTP (0.2 mM), CTP (0.2 mM), [5′,6′-^{3}H]UTP (0.45 μM, 0.02 TBq mmol^{-1}), each enzyme (10 μL), and denatured calf thymus DNA (0.17 μg). The reaction was incubated at 30 °C. After running the reaction for the indicated times, the reaction was stopped by the addition of SDS solution (10 μL, 1%) containing sodium pyrophosphate (50 mM). An aliquot (15 μL) was deposited on a DEAE cellulose filter (DE81, Whatman), and nucleotide triphosphates not incorporated into RNA were removed by washing the filter five times with Na_2HPO_4 (5%), twice with water, and finally twice with EtOH. Incorporation of [5′,6′-^{3}H]UTP into the synthesized RNA was measured using a liquid scintillation counter (Perkin–Elmer). ppGpp was added to the reaction solution just before starting the reaction.

Cross-linking: Radioactive 6-thioppGpp was added to a solution containing HEPES (25 mM, pH 7.9), $MgCl_2$ (10 mM), KCl (100 mM), bovine serum albumin (25 μg mL^{-1}), and chloroplast RNAP (20 μg mL^{-1}) in a microtube (1.5 mL). The reaction mixture was incubated for 1 h at 30 °C and then transferred to ice and irradiated with UV light (365 nm, 743 μW cm^{-2}) for 30 min at a distance of 4 cm. After cross-linking, the reaction was stopped by the addition of 4× SDS sample buffer [0.25 M Tris-HCl (pH 6.8), 8% sodium dodecyl sulfate, 20% 2-mercaptoethanol, 0.01% bromophenol blue, 40% glycerol] to a 1× concentration. Samples were placed at room temperature for 30 min, and then resolved by a SDS-PAGE electrophoresis. Proteins were stained by Silver stain kit (Wako) and radioactive 6-thioppGpp cross-linked proteins were further visualized by autoradiography.

Protein identification using mass spectrometry: The protein band of interest in cross-linking experiment was excised from CBB-stained gels. The gel pieces were thoroughly destained with $(NH_4)_2CO_3$ (25 mM) in acetonitrile aqueous solution (50%) and dried under vacuum. Trypsin (mass spectrometry grade, Promega) was added and the tube was placed on ice for 30 min. After removing the extra solution, the tube was then placed at 37 °C for 12 h for tryptic digestion of proteins. The gel pieces were extracted with acetonitrile aqueous solution (50%) containing trifluoroacetic acid (0.1%). Peptide extract was concentrated to 10 μL in vacuo, desalted using a ZipTipC18 (Millipore), and analyzed by MALDI-TOF mass spectrometry using 2,5-dihydroxy benzoic acid as a matrix in a reflector mode. Identification of the protein from MALDI-TOF peptide mass fingerprint data was performed using the MASCOT search program (http://www.matrixscience.com).

Transcription assay of chloroplast nucleoids and proplastid nucleoids: Mature leaves of tobacco plants (*Nicotiana tabacum* L. cv. Bright Yellow-2) were used for this experiment. The culture conditions of the tobacco cell line BY-2, derived from the same cultivar, and isolation of chloroplast nucleoids and proplastid nucleoids were carried out according to the methods of Sakai et al.[40] The transcriptional activities of isolated chloroplast nucleoids and proplastid nucleoids were assayed as described above.

Acknowledgements

We thank K. Watanabe and Dr. E. Fukushi for measuring mass spectral data of synthesized nucleotides. We also thank Dr. S. Hamada for providing BY-2 cells and Japan Tobacco Inc. for providing seeds of N. tabacum *L. cv. Bright Yellow-2. This work was supported by a Grant-in-Aid for Scientific Research (C) 19580118 from the Japan Society for the Promotion of Science (JSPS) to K.T.*

Keywords: chloroplast · gene expression · ppGpp · RNA polymerase · RNA · stringent response

[1] "The Stringent Response", M. Cashel, D. R. Gentry, V. J. Hernandez, D. Vinella In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology, 2nd Ed.* (Eds.: F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Renznikoff, M. Riley, M. Shcaechter, H. E. Umbarger) American Society for Microbiology, Washington D.C. **1996**, pp. 1458–1496.
[2] K. Ochi, *J. Bacteriol.* **1987**, *169*, 3608–3616.
[3] T. Inaoka, K. Takahashi, M. Ohnishi-Kameyama, M. Yoshida, K. Ochi, *J. Biol. Chem.* **2003**, *278*, 2169–2176.
[4] B. Z. Harris, D. Kaiser, M. Singer, *Genes Dev.* **1998**, *12*, 1022–1035.
[5] C. van Delden, R. Comte, A. M. Bally, *J. Bacteriol.* **2001**, *183*, 5376–5384.
[6] S. Haralalka, S. Nandi, R. K. Bhadra, *J. Bacteriol.* **2003**, *185*, 4672–4682.
[7] A. Calderón-Flores, G. Du Pont, A. Huerta-Saquero, H. Merchant-Larios, L. Servín-González, S. Durán, *J. Bacteriol.* **2005**, *187*, 5075–5083.
[8] K. Braeken, M. Moris, R. Daniels, J. Vanderleyden, J. Michiels, *Trends Microbiol.* **2006**, *14*, 45–54.
[9] D. Chatterji, A. K. Ojha, *Curr. Opin. Microbiol.* **2001**, *4*, 160–165.
[10] M. Sugiura, *Plant Mol. Biol.* **1992**, *19*, 149–168.
[11] C. Lemieux, C. Otis, M. Turnel, *Nature* **2000**, *403*, 649–652.
[12] E. A. Lysenko, V. V. Kuznetsov, *Mol. Biol.* **2005**, *39*, 661–674.
[13] T. Shiina, Y. Tsunoyama, Y. Nakahira, M. S. Khan, *Int. Rev. Cytol.* **2005**, *244*, 1–68.
[14] T. Pfannschmidt, K. Ogrzewalla, S. Baginsky, A. Sickmann, H. E. Meyer, G. Link, *Eur. J. Biochem.* **2000**, *267*, 253–261.
[15] J. Y. Suzuki, A. J. Ytterberg, T. A. Beardslee, L. A. Allison, K. J. Wijk, P. Maliga, *Plant J.* **2004**, *40*, 164–172.
[16] J. Pfalz, K. Liere, A. Kandlbinder, K. J. Dietz, R. Oelmüller, *Plant Cell* **2006**, *18*, 176–197.
[17] B. Hedtke, T. Börner, A. Weihe, *Science* **1997**, *277*, 809–811.
[18] B. Hedtke, T. Börner, A. Weihe, *EMBO Rep.* **2000**, *1*, 435–440.
[19] J. Hu, L. Boqorad, *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 1531–1535.
[20] T. Pfannschmidt, G. Link, *Plant Mol. Biol.* **1994**, *25*, 69–81.
[21] E. A. van der Biezen, J. Sun, M. J. Coleman, M. J. Bibb, J. D. Jones, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 3747–3752.
[22] K. Kasai, S. Usumi, T. Yamada, Y. Endo, K. Ochi, Y. Tozawa, *Nucleic Acids Res.* **2002**, *30*, 4985–4992.
[23] A. Yamada, K. Tsutsumi, S. Tanimoto, Y. Ozeki, *Plant Cell Physiol.* **2003**, *44*, 3–9.
[24] R. M. Givens, M.-H. Lin, D. J. Taylor, U. Mechold, J. O. Berry, V. J. Hernandez, *J. Biol. Chem.* **2004**, *279*, 7495–7504.
[25] K. Takahashi, K. Kasai, K. Ochi, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4320–4324.
[26] Y. Tozawa, A. Nozawa, T. Kanno, T. Narisawa, S. Masuda, K. Kasai, H. Nanamiya, *J. Biol. Chem.* **2007**, *282*, 35536–35545.
[27] S. Masuda, K. Mizusawa, T. Narisawa, Y. Tozawa, H. Ohta, K. Takamiya, *Plant Cell Physiol.* **2008**, *49*, 135–141.
[28] D. Chatterji, N. Fujita, A. Ishihama, *Genes Cells* **1998**, *3*, 279–287.
[29] I. I. Toulokhonov, I. Shulgina, V. J. Hernandez, *J. Biol. Chem.* **2001**, *276*, 1220–1225.
[30] I. Artsimovitch, V. Patlan, S. Sekine, M. Vassylyeva, T. Hosaka, K. Ochi, S. Yokoyama, D. Vassylyev, *Cell* **2004**, *117*, 299–310.
[31] H. J. Breter, M. Harald, *Biochim. Biophys. Acta* **1990**, *1033*, 124–132.
[32] C. Schmitz-Linneweber, R. M. Maier, J. P. Alcaraz, A. Cottet, R. G. Herrmann, R. Mache, *Plant Mol. Biol.* **2001**, *45*, 307–315.
[33] M. M. Barker, T. Gaal, C. A. Josaitis, R. L. Gourse, *J. Mol. Biol.* **2001**, *305*, 673–688.
[34] K. Kasai, T. Nishizawa, K. Takahashi, T. Hosaka, H. Aoki, K. Ochi, *J. Bacteriol.* **2006**, *188*, 7111–7122.
[35] B. J. Paul, M. M. Barker, W. Ross, D. A. Schneider, C. Webb, J. W. Foster, R. L. Gourse, *Cell* **2004**, *118*, 311–322.
[36] P. Maliga, *Trends Plant Sci.* **1998**, *3*, 4–6.
[37] L. A. Allison, *Biochimie* **2000**, *82*, 537–548.
[38] G. M. Hudson, T. A. Holton, P. R. Whitfeld, W. Bottomley, *J. Mol. Biol.* **1988**, *200*, 639–654.
[39] A. Sakai, C. Saito, N. Inada, T. Kuroiwa, *Plant Cell Physiol.* **1998**, *39*, 928–934.
[40] A. Sakai, T. Suzuki, Y. Miyazawa, T. Kuroiwa, *Plant Sci.* **1998**, *133*, 17–31.
[41] E. A. Lysenko, *Plant Cell Rep.* **2007**, *26*, 845–859.
[42] S. E. Perry, H. M. Li, K. Keegstra, *Methods Cell Biol.* **1991**, *34*, 327–344.
[43] S. Lerbs, J. F. Briat, R. Mache, *Plant Mol. Biol.* **1983**, *2*, 67–74.

Received: November 11, 2008
Published online on March 23, 2009

