Calcium-activated (p)ppGpp Synthetase in Chloroplasts of Land Plants*

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The genetic system of chloroplasts, including the machinery for transcription, translation, and DNA replication, exhibits substantial similarity to that of eubacteria. Chloroplasts are also thought to possess a system for generating guanosine 5′-triphosphate ((p)ppGpp), which triggers the stringent response in eubacteria, with genes encoding chloroplastic (p)ppGpp synthetase having been identified. We now describe the identification and characterization of genes (OsCRSH1, OsCRSH2, and OsCRSH3) for a novel type of (p)ppGpp synthetase in rice. The proteins encoded by these genes contain a putative chloroplast transit peptide at the NH2 terminus, a central RelA-SpoT-like domain, and two EF-hand motifs at the COOH terminus. The recombinant OsCRSH1 protein was imported into chloroplasts in vitro, and genetic complementation analysis revealed that expression of OsCRSH1 suppressed the phenotype of an Arabidopsis thaliana, indicating that such genes are conserved among both monocotyledonous and dicotyledonous land plants. CRSH proteins thus likely function as Ca2+-activated (p)ppGpp synthetases in plant chloroplasts, implicating both Ca2+ and (p)ppGpp signaling in regulation of the genetic system of these organelles.

Guanosine 5′-triphosphate (or 5′-diphosphate) 3′-diphosphate ((p)ppGpp) is an effector molecule responsible for the stringent response, a global regulatory system in bacteria (1, 2).

The hyperphosphorylated guanosine nucleotides ppGpp and pppGpp were initially identified as “magic spots” that accumulate during amino acid deprivation in Escherichia coli and induce rapid down-regulation of stable RNA synthesis (1). Subsequently, carbon, fatty acid, or iron limitation was also found to trigger the accumulation of (p)ppGpp in E. coli (2–5). Specific targets of the global regulator (p)ppGpp have been identified among enzymes of nucleotide metabolism (6) and protein components of the apparatuses for transcription (7), translation (8), and DNA replication (9).

Two enzymes, RelA and SpoT, are responsible for fluctuations in (p)ppGpp concentration in E. coli. RelA is a ribosome-associated protein and synthesizes (p)ppGpp in response to the increase in the proportion of uncharged tRNAs that results from amino acid starvation (10). In contrast, SpoT is a bifunctional (p)ppGpp synthetase and hydrolase and regulates (p)ppGpp levels in response to carbon source or fatty acid limitation (3, 4, 11). Many other bacteria possess only a single bifunctional (p)ppGpp synthetase and hydrolase, designated Rel (12). Rel proteins have been shown to be essential for the long term survival or virulence of pathogenic bacteria (13).

Stringent factor and RelA-SpoT-like (p)ppGpp synthetases have also been identified in chloroplasts (14–17), and we previously demonstrated the presence of intrinsic (p)ppGpp synthetase activity in pea chloroplasts (18). Three RelA-SpoT homolog genes (RSH1, RSH2, and RSH3) have been identified in plants (14, 17), and one such gene (RSH) has been identified in the alga Chlamydomonas reinhardtii (16). Enzymatic activity and specific localization to chloroplasts have been demonstrated for plant RSH2 and RSH3 and for algal RSH (16, 17). Chloroplasts of land plants and algae are thought to originate from ancient cyanobacteria-like prokaryotes. Indeed, the genetic system of chloroplasts, including the apparatuses for transcription, translation, and DNA replication, is highly similar to that of bacteria. A stringent control system similar to that of bacteria has therefore been suggested to operate in chloroplasts of photosynthetic eukaryotes (19).

In a search for plant genes that encode RelA-SpoT-like proteins, we have now identified a previously unknown type of RSH gene for putative chloroplast-localizing proteins in rice (Oryza sativa). The deduced amino acid sequences of the novel RSH genes contain motifs similar to the EF-hand, a Ca2+-binding domain, at their COOH termini. We show that the (p)ppGpp synthetase activity of the gene products is regulated by Ca2+ via...
the EF-hand motifs, and we therefore named them CRSH (Ca$^{2+}$-activated RelA-SpoT homolog) to distinguish their mechanism of regulation from that of other RSH proteins. Our findings thus have implications for Ca$^{2+}$ signaling and Ca$^{2+}$-dependent (p)ppGpp accumulation in plant chloroplasts.

**EXPERIMENTAL PROCEDURES**

**Plant and Bacterial Materials**—Rice (O. sativa cv. Nipponbare) plants were grown as described previously (20). Pea (Pisum sativum cv. Toyonari; Sakata Seed Co., Yokohama, Japan) plants were grown at 25 °C under a 16-h light, 8-h dark cycle with artificial light. E. coli strains W3110 (wild type), CF1652 (relA$^-$), and CF1678 (relA$^{-}$, spoT$^{-}$) were used for genetic complementation analysis (16).

**Isolation and Characterization of Rice CRSH Genes**—Standard recombinant DNA techniques were performed basically as described (21). The PCR was performed with a rice cDNA library (20) and oligonucleotide primers (OsREL1F, 5'-CAGG-GAGGACACAAAAATATATGTCAC-3'; OsREL1R, 5'-CGTC-GAGAAGATGCATGAG-3') based on a rice expressed sequence tag that shows sequence similarity to bacterial relA and spoT (GenBankTM accession number D48993). The PCR products were cloned into the pCRII vector (Invitrogen) and sequenced. Amplified DNA fragments were also purified and used to prepare fluorescein isothiocyanate-labeled probes with an ECL random prime labeling system (GE Healthcare). Screening of a Nipponbare cDNA library in phage Agt11 for OsCRSH1 cDNA clones and isolation of such clones were performed as previously described (20).

Complementary DNA for OsCRSH3 was constructed as follows. The cDNA clone AK110850, which encodes an NH$_2$-terminal portion of OsCRSH3, was obtained from the National Institute of Agricultural Resources (NIAR, Tsukuba, Japan). The missing portion of the open reading frame (ORF) was predicted from the corresponding genomic sequence of rice chromosome 5 (accession number AC137614). The 1598-bp portion of the ORF contained in clone AK110850 was amplified by PCR with the forward primer CRSH3F (5'-ATAACTAGTAT-GGCCAACCGCGGTTCA-3') and the reverse primer CRSH3R (5'-ATGATGGCCCTAGCCCTTT-3'). The portion of the ORF for the COOH-terminal region of OsCRSH3 was amplified by PCR with a template cDNA mixture (20) prepared from 20-day-old Nipponbare seedlings, the forward primer CR3MF (5'-GTTGATCAAGGCCATGGA-3'), and the reverse primer CR3MR (5'-AAAGTCGACTTAGCTACAAGCTTG-3'). The new PCR products were then subjected to a further PCR amplification with the primers CR3MF and CR3R (5'-ATAAGATCTTCAAACGAGGACCAACTTGTG-GGCGAACGCCGGTGTCAA-3'; CR3MR, 5'-CCTCTTCACAAACGAGC-AAT-3'), for OsCRSH2 (CR3HF, 3'-CAGG-GAGGACACAAAAATATATGTCAC-3'; CR3HR, 5'-CCTCTTCACAAACGAGCA-AATT-3'), for OsCRSH1 (CRSH1F, 5'-CATGTGGCCATCGTGAAG-3'; CRSH1R, 5'-CCGCTCCGTAGCATTG-3'); OsCRSH3 (CRSH3F, 5'-CATGTGGCCATCGTGAAG-3'; CRSH3R, 5'-CCGCTCCGTAGCATTG-3'). As a control, PCR was also performed with plasmids containing OsCRSH1, OsCRSH2, or OsCRSH3 cDNAs as templates and with the corresponding primer sets. The reaction mixture (50 μl) contained 1× ExTaq buffer, 1 μg of cDNA, 0.2 mM of each deoxynucleoside triphosphate, 1 μM of each primer, and 2.5 units of ExTaq DNA polymerase (TaKaRa, Shiga, Japan). Amplification was performed in a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA) with an initial denaturation step of 94 °C for 5 min followed by 25, 30, 35, or 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 20 s. A portion (5 μl) of the reaction mixture was then analyzed by electrophoresis through a 3% agarose gel and staining of DNA with ethidium bromide.

**Cell-free Protein Synthesis**—A series of cDNAs encoding intact or NH$_2$-terminally truncated forms of OsCRSH1 was constructed by PCR with a plasmid harboring the full-length cDNA as template, a gene-specific forward primer (FNF for the full-length protein, 5'-CCATTGCGCAGCGCAGCG-3'); DN41F for a protein lacking the N41-terminal amino acids, 5'-CCATGGCCCAAGCAGCCGCCGCG-3'; DN41F for a protein lacking the 65 NH$_2$-terminal residues, 5'-CCATTGCGCAGCGCAGCG-3'); and the reverse primer CR5R (5'-AAAGTCGACTTAGCTACAAGCTTG-3'). The amplified fragments were digested with Ncol and Sall and then cloned into the corresponding sites of pEU3b. The resulting plasmids were designated pECR1FL, pECR1ΔN41, and pECR1ΔN65, respectively. A pEU3b-based vector for an OsCRSH1 mutant lacking both the 41 NH$_2$-terminal and 128 COOH-terminal residues was similarly constructed by PCR with the plasmid encoding the full-length protein, the forward primer DN41F, and the reverse primer DC128. After verification of the inserted sequences, the various plasmids for the truncated proteins were used as templates for in vitro transcription with SP6 RNA polymerase as described (23).

For the synthesis of glutathione S-transferase (GST) fusion proteins of OsCRSH1ΔN41 or OsCRSH1ΔN41ΔC128, a DNA fragment encoding GST followed by the amino acid sequence Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro, which is the rec-
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Omission site of Precision protease (GE Healthcare), was first prepared by PCR with the plasmid pKT7GST2 (24) as template, the forward primer GSTF-Sac (5’-GAGCTCATATGCCCCTCAT-TACTAGTTATGG-3’), and the reverse primer GSTR-Sma (5’-CCCGGGGCCCCCTGGAACAGAACTTTCCAGCTT-3’). The PCR product was subcloned into the pcRII vector and sequenced, and the verified SacI-Smal fragment was then cloned into the corresponding sites of pEU3b to yield pEGST. OsCRSH1 cDNAs were constructed by PCR with pECR1ΔN41 or pECR1ΔN41AC128 as the template, the genespecific forward primer DN41F (5’-CCCAGGCTGGGAGACGAGCTTT-3’), and the reverse primer CR2R (5’-CATGTATGGCGGCATGG-3’). For OsCRSH2 and OsCRSH3, cDNA fragments corresponding to the putative mature form of each protein were amplified by PCR with a plasmid containing the full-length ORF (clone AK058438 for OsCRSH2; pECRSH3 for OsCRSH3), the forward primer CR2DN14F (5’-ATAACTAGTATGGCGGCACGAGTGC-3’) for OsCRSH2 or CR3DN12F (5’-ATAACTAGTATGGCGGCAGGACGAC-3’) for OsCRSH3, and the reverse primer CR2R (5’-ATAAGATCTTGCGGGAGAGCAGCTT-3’) for OsCRSH2 or CR3R for OsCRSH3. The PCR products were digested with SpeI and cloned into the Smal-SalI sites of pEGST. The resulting plasmids were designated pGCR1ΔN41 and pGCR1ΔN41ΔC128, respectively.

For construction of cell-free expression vectors for OsCRSH2 and OsCRSH3, cDNA fragments corresponding to the putative mature form of each protein were amplified by PCR with a plasmid containing the full-length ORF (clone AK058438 for OsCRSH2; pECRSH3 for OsCRSH3), the forward primer CR2DN14F (5’-ATAACTAGTATGGCGGCACGAGTGC-3’) for OsCRSH2 or CR3DN12F (5’-ATAACTAGTATGGCGGCAGGACGAC-3’) for OsCRSH3, and the reverse primer CR2R (5’-ATAAGATCTTGCGGGAGAGCAGCTT-3’) for OsCRSH2 or CR3R for OsCRSH3. The PCR products were digested with SpeI and BgIII and cloned into the corresponding sites of pEU3b, generating pECRSH2ΔN14 and pECRSH3ΔN12, respectively.

Cell-free protein synthesis was performed with wheat embryo extracts (Cell-Free Sciences, Yokohama, Japan) according to two different methodologies. For the synthesis of 35S-labeled proteins, the “batch method” was performed as described (23) in the presence of [35S]methionine and [35S]cysteine (total of 14.8 mCi/ml) with the use of a Pro-mixL-[35S] in vitro cell labeling kit (GE Healthcare). For preparation of protein for enzyme assays, the “bilayer method” (22) was performed. GST-OsCRSH1 fusion proteins synthesized by the bilayer method were isolated on a glutathione affinity column to yield the corresponding OsCRSH1 protein. The solubility of proteins synthesized in the presence of [14C]leucine (0.074 mCi/ml; GE Healthcare) was assessed by SDS-PAGE both of the total reaction mixture and of the supernatant obtained after centrifugation of the mixture at 22,000 × g for 20 min at 4 °C. The gel was dried and exposed to a BAS-III imaging plate (FujiFilm, Tokyo, Japan), and protein-associated radioactivity was detected with a BAS-2500 analyzer (FujiFilm) and quantitated with Image Gauge version 3.41 software (FujiFilm).

Site-directed Mutagenesis—Site-directed mutagenesis of OsCRSH1 was performed by in vitro overlap extension PCR as described (25). The gene was mutated so that glutamate in the Z position of either Ca2+ binding loop (see Fig. 1C) was replaced with glutamine. Two separate amplification reactions were performed to amplify the two halves of OsCRSH1 with the use of four primers. The outside-forward primer (DN41FB) was paired with a middle-reverse mutation primer (EF1R for the NH2-terminal Ca2+ binding loop, 5’-CCTCCTGTGAGCTGCTCAATACTGAT-3’, with the substituted nucleotide underlined; EF2R for the COOH-terminal Ca2+ binding loop, 5’-GAATGCTTCGATTGATCGAGCTCAAGG-3’) to generate the first half of the gene; the outside-reverse primer (FCR) was paired with a middle-forward mutation primer (EF1F for the NH2-terminal Ca2+ binding loop, 5’-ATCGATTTAGGAGCTCTACAGGG-3’, EF2F for the COOH-terminal Ca2+ binding loop, 5’-CTTGAGCTCCGATCAATTTGAGCATCCC-3’) to generate the second half. The reactions were performed with 5 ng of pECR1ΔN41 as the template in a final volume of 50 μl containing 1× Pyrobest Buffer II (TaKaRa), 0.2 mm of each deoxynucleoside triphosphate, 0.025 unit of Pyrobest DNA polymerase (TaKaRa), and 0.2 μm of each primer. The amplification protocol comprised 20 cycles of denaturation at 98 °C for 15 s, annealing at 60 °C for 35 s, and elongation at 72 °C for 3 min, followed by a final elongation step at 72 °C for 10 min. The DNA fragments bearing overlapping sequences introduced by the two middle primers were then mixed together and subjected to three cycles of denaturation at 98 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 3 min. The resulting products were further amplified by PCR with the outside-forward primer (DN41FB) and the outside-reverse primer (FCR); the protocol comprised 20 cycles of denaturation at 98 °C for 15 s, annealing at 60 °C for 35 s, and elongation at 72 °C for 3 min, followed by a final elongation step at 72 °C for 10 min. The final products were digested with SalI and cloned into the Smal-Sall sites of pGCR1ΔN41. The resulting plasmids for GST fusion proteins of OsCRSH1(ΔN41) with mutated NH2- and COOH-terminal Ca2+-binding loops were designated pGCR1ΔN41EF1 and pGCR1ΔN41EF2, respectively.

Complementation Test of OsCRSH1 in E. coli—The E. coli strains W3110, CF1652 (relA+), and CF1678 (relA-, spoT-) were prepared as recipients of test plasmids by lysogenic introduction of λ-DE3 phage (EMD Biosciences, San Diego, CA) into their chromosomal DNA to confer IPTG-inducible gene expression from the T7 promoter (16). For construction of an expression vector for OsCRSH1(ΔN41), the full-length OsCRSH1 cDNA was subjected to PCR with the primers DN41F and CR1RB (5’-GGATCCTATTAGCCTACAGCAGATTGTTCGCCC-3’) with mutated NH2- and COOH-terminal Ca2+-binding loops. The resulting product was digested with Ncol and BamHI and substituted for the Ncol-BamHI region of pET11d (Novagen, Darmstadt, Germany), yielding pETCR1. The cells were transformed with pETCR1 or pET11d, transferred to MOPS agar medium (40 mM MOPS and 4 mM Tricine buffer (adjusted to pH 7.2 with KOH), 50 mM KCl, 10 mM NH4Cl, 0.5 mM MgSO4, 2 mM KH2PO4, 10 mM FeCl3, 1.6% agar) (16) containing 0.4% glucose with or without 50 μM IPTG, and grown at 37 °C for 24 h.

Assay of (p)ppGpp Synthetase Activity—ATP:GTP 5’-pyrophosphotransferase assays were performed in a final volume of 25 μl containing 2 mM ATP, 1 μCi [32P]ATP at 10 μCi/ml (3000 Ci/nmol; GE Healthcare), 1.3 mM GTP, and synthesized protein in Buffer C (50 mM Tris acetate, pH 7.8, 0.5 mM magnesium acetate, 60 mM potassium acetate, 30 mM ammonium acetate, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and in the absence or presence of CaCl2. The reaction was performed for 1 h at 30 °C and was stopped by the addition of 1 μl of 88% formic acid. After the further addition of 12.5 μl of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), the mix-
ture was agitated and then centrifuged at 10,000 × g for 5 min at 4 °C. The aqueous phase was transferred to another tube, and a 3-μl portion was spotted onto a polyethyleneimine-cellulose thin layer sheet (Merck). For one-dimensional analysis, 1.5 M KH₂PO₄ was used as the chromatographic solvent, as described (18). Two-dimensional analysis was performed essentially as described previously (18) with solvent T (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 (NH₄)₂SO₄, 0.4 g of (NH₄)H₂SO₄, and 4 g of EDTA (disodium salt) in 100 ml of H₂O) for the second dimension. Each chromatogram was exposed to a BAS-III imaging plate, and the associated radioactivity was detected with a BAS-2500 analyzer and quantitated with Image Gauge version 3.41 software.

**Assay of (pppGpp) Hydrolase Activity**—The 32P-labeled pppGpp was synthesized in vitro by using purified OsCRSH1ΔN41 in the presence of 500 μM CaCl₂ as described in the method for (pppGpp synthetase assay. Purification of the 32P-labeled pppGpp was carried out as described (18). Hydrolysis mixtures (25 μl) contained 50 mM Tris acetate, pH 7.8, 0.5 mM magnesium acetate, 60 mM potassium acetate, 30 mM ammonium acetate, 1 mM dithiothreitol, 0.5 μg of enzyme, and 3 μl of the purified pppGpp with or without the addition of 1 mM MnCl₂. The mixtures were incubated at 30 °C for 2 h, and the reaction was stopped by the addition of 1 μl of 88% formic acid. After the further addition of 12.5 μl of phenol:chloroform: isoamylalcohol (25:24:1, v/v/v), the mixture was agitated and then centrifuged at 10,000 × g for 5 min at 4 °C. The aqueous phase was transferred to another tube, and a 5-μl portion was subjected to the one-dimensional TLC analysis.

**Expression and Purification of SpoT Protein**—A DNA segment of E. coli spoT gene coding for the NH₂-terminal fragment (1–380 amino acids) of the SpoT protein was amplified by PCR using primers SPQEF (5’-GGATCCCTTGATCTGGTTGGA-AGCCCTGAA-3’) and SPQER (5’-AACGCACTACCGGCCTGT-3’). The obtained DNA fragment was digested with BamHI and HindIII and cloned into the BamHI-HindIII sites of pQE30 (Qiagen), yielding pQESPΔC. E. coli strain JM109 transformed with pQESPΔC was incubated in 100 ml of LB at 37 °C with vigorous shaking. When the optical density (600 nm) was reached at 0.5, IPTG was added to the culture at a final concentration of 1 mM and further incubated at 37 °C for 3 h. The cells were collected by centrifugation at 6,000 × g for 15 min at 4 °C and then resuspended in TALON buffer A (50 mM sodium phosphate, pH 7.0, 0.5 M NaCl, 17 mM imidazole, 0.1% Triton X-100, 20% glycerol). The cells were lysed by sonication, and the cell debris was removed by centrifugation at 12,000 × g for 15 min at 4 °C. The lysate was added to TALON Metal affinity resins (Clontech, Mountain View, CA) for 1 h at room temperature, and the resin was then transferred to a column and washed with TALON wash buffer (50 mM sodium phosphate, pH 7.0, 0.5 M NaCl, 17 mM imidazole, 0.1% Triton X-100, 20% glycerol). The SpoTΔC protein tagged with 6×His at its NH₂-terminus was eluted from the column with TALON elution buffer (50 mM sodium phosphate, pH 7.0, 0.5 M NaCl, 180 mM imidazole, 0.1% Triton X-100, 20% glycerol). The purity was checked by SDS-PAGE analysis, and the fraction containing SpoTΔC was stored until use at −30 °C after the addition of equal volume of 100% glycerol.

**RESULTS**

**Isolation and Characterization of OsCRSH cDNAs**—In a search for (pppGpp synthetase genes in land plants, we found a rice expressed sequence tag (GenBank™ accession number D48993) that encodes part of a putative polypeptide with sequence similarity to RelA and SpoT of E. coli, and we subsequently isolated a cDNA containing the corresponding full-length ORF from a rice cDNA library in λgt11 (20). After we deposited the sequence of this cDNA in DDBJ (GenBank™ accession number AB042936), the same cDNA sequence appeared (AK121808) in the rice full-length cDNA database (cdna01.dna.affrc.go.jp/cDNA). A data base search identified a similar cDNA sequence in Arabidopsis thaliana (At3g17470). The predicted proteins encoded by the rice and Arabidopsis cDNAs each comprise 583 amino acids and show sequence similarity to RelA-SpoT family proteins (Fig. 1A). To compare the deduced amino acid sequences of the plant proteins with those of other RelA-SpoT family members, we aligned the regions implicated in (p)ppGpp hydrolase and (p)ppGpp synthetase activities (Fig. 1B). The plant proteins also include an NH₂-terminal extension predicted to contain a chloroplast-targeting signal as well as two EF-hand motifs in the COOH-terminal region (Fig. 1, A and C). On the basis of these structural features and subsequent functional characterization of the encoded protein, we designated the isolated rice gene as OsCRSH1 (Q. sativa Ca²⁺-activated RelA-SpoT homolog 1). A further search resulted in the identification of two additional homologous genes (GenBank™ accession numbers AK058438 and AK110850) in the rice expressed sequence tag data base. Whereas AK058438 contains a full-length ORF, the sequence of AK110850 was annotated as a cDNA in the reverse direction, with the DNA sequence corresponding to the COOH-terminal region of the encoded protein being missing. Given that the genomic sequence of rice chromosome 5 contains the genes corresponding to AK058438 and AK110850, we isolated the full-length cDNAs for both genes by PCR from a cDNA mixture prepared from polyadenylated RNA of rice leaves. The deduced amino acid sequences of both genes include a putative chloroplast-targeting signal in the NH₂-terminal region, a central RelA-SpoT-like domain, and EF-hand motifs in the COOH-terminal region (Fig. 1). These two genes were thus designated OsCRSH2 (GenBank™ accession number AK058438) and OsCRSH3 (GenBank™ accession number AB298325). The extent of nucleotide sequence identity between OsCRSH1 and either OsCRSH2 or OsCRSH3 cDNAs is 66.3 and 65.3%, respectively, whereas the corresponding values for amino acid sequence identity are 62.4 and 64.8%. All three genes are present on rice chromosome 5. An unrelated gene (expressed sequence tag clone AK105485) is positioned between OsCRSH1 and OsCRSH2 (data not shown). The genome sequence of A. thaliana appears to contain only a single gene, located on chromosome 2, that is homologous to the OsCRSH genes. It is therefore likely that the three OsCRSH genes arose as a result of triplication of the ancestral gene during rice evolution.
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**A**

![Diagram of domain organization of plant CRSH proteins](image)

**B**

| Protein | Sequence |
|---------|----------|
| OsCRSH1 | 81 NBLTCHEGGAGTSGRKLPRKAFCDGFVGRYAAKAKGAGAA |
| OsCRSH2 | 127 AAGATAGCPCVAVDFGDLQKAFCDGFVGRYAAKAKGAGAA |
| AtCRSH  | 120 RVRVEISGLYKSHCPRKAFCDGFVGRYAAKAKGAGAA |
| AtRSH2  | 137 MRRKPDLDHGKSDGGTGFCDGFVGRYAAKAKGAGAA |
| NtRSH2  | 200 AEGHLKDDHQKSDGGTGFCDGFVGRYAAKAKGAGAA |
| SpoT    | 4 FESNLQYTFDDRQDDDFCDGFVGRYAAKAKGAGAA |
| CrRSH   | 178 PLGAHNFVAVDQTHDQDDDFCDGFVGRYAAKAKGAGAA |
| RelA    | 15 WAGHYDQRDDDFCDGFVGRYAAKAKGAGAA |

**C**

| Protein | Sequence |
|---------|----------|
| OsCRSH1 | 157 RDKTDFMGRKAFCDGFVGRYAAKAKGAGAA |
| OsCRSH2 | 200 TVTSTFMTKAFCDGFVGRYAAKAKGAGAA |
| AtCRSH  | 122 RVRVEISGLYKSHCPRKAFCDGFVGRYAAKAKGAGAA |
| AtRSH2  | 225 ILSRADGCCGKAFCDGFVGRYAAKAKGAGAA |
| NtRSH2  | 350 AEGHLKDDHQKSDGGTGFCDGFVGRYAAKAKGAGAA |
| SpoT    | 275 DYNRFFQDDDFCDGFVGRYAAKAKGAGAA |
| CrRSH   | 255 QDLMQVFVAVDQTHDQDDDFCDGFVGRYAAKAKGAGAA |
| RelA    | 191 WAGHYDQRDDDFCDGFVGRYAAKAKGAGAA |

**FIGURE 1.** Structural characteristics of plant CRSH proteins. **A**, domain organization of plant CRSH. The NH2-terminal region contains a chloroplast transit peptide (cTP); the central region contains a RelA-SpoT-like domain including both (p)pGpp hydrolysis (Hyd) and (p)pGpp synthetase (Syn) motifs; and the COOH-terminal regions contains two typical EF-hand motifs. **B**, alignment of the deduced amino acid sequences of the RelA-SpoT-like domain of plant CRSH proteins with those of the corresponding domains of *A. thaliana* RSH2 and RSH3 (AtRSH2 and AtRSH3), *Nicotiana tabacum* RSH2 (NtRSH2), *E. coli* SpoT (SpoT), *C. reinhardtii* RSH (CrRSH), and *E. coli* RelA (RelA). Hyphens indicate gaps introduced to optimize alignment. Identical or similar residues among the various proteins are indicated by dark and light shading, respectively, and residue numbers are shown on the left and at the end of the sequences. The dashed and solid arrows correspond to the Hyd and Syn regions indicated in **A**, respectively. **C**, comparison of the deduced amino acid sequences of the EF-hand motifs of the three rice CRSH proteins (OsCRSH1, OsCRSH2, and OsCRSH3) and *Arabidopsis* CRSH (AtCRSH). Conserved residues in the Ca2⁺-binding loops are shown in bold, and the residue numbers are shown on the left. The regions corresponding to E helices, Ca2⁺-binding loops, and F helices are indicated by bars, with the consensus sequences for these regions being shown below the bars. h, hydrophobic amino acid; *, any amino acid; X, Y, Z, −Y, −X, and −2, amino acids that chelate Ca2⁺.
Localization and Processing of OsCRSH1—To examine the function of the NH₂-terminal sequence of the putative OsCRSH1 protein, we performed an in vitro chloroplast import assay as described previously (26). The full-length protein was synthesized with a wheat embryo cell-free protein synthesis system in the presence of [35S]methionine and [35S]cysteine. Incubation of the 35S-labeled protein (∼64 kDa) with intact pea chloroplasts resulted in its processing to a smaller form (∼60 kDa) that was resistant to incubation with the protease thermolysin (Fig. 2A). The resistance of processed protein to thermolysin indicates that mature OsCRSH1 protein was taken up into chloroplasts. These results thus indicated that OsCRSH1 was imported into chloroplasts, likely as a result of its putative NH₂-terminal signal sequence (Fig. 1A).

We next compared the molecular size of the processed form of OsCRSH1 with that of NH₂-terminal truncation mutants also synthesized with the cell-free translation system. The size (∼60 kDa) of the processed form of OsCRSH1 was similar to that of an OsCRSH1 mutant lacking the 41 NH₂-terminal amino acids (Fig. 2B), suggesting that the cleavage site is located near the residue, alanine 42, in the polypeptide sequence (AB042936). The NH₂-terminal sequence of the OsCRSH1(ΔN41) mutant is also similar to that of the predicted cleaved form of the Arabidopsis AtCRSH protein (cleavage site between arginine 15 and serine 16, At3g17470). On the basis of these results, we used the ΔN41 form of OsCRSH1 as the mature form of the protein in subsequent experiments.

Functional Complementation of an E. coli Mutant by OsCRSH1—To determine whether OsCRSH1 functions as a RelA-SpoT homolog, we cloned the cDNA encoding the putative mature form of the protein into the expression vector pET11d to yield pETCR1. Transformation of E. coli CF1678(DE3) (relA⁻, spoT⁻) and W3110 (wild type) were transformed with pETCR1 (encoding OsCRSH1(ΔN41)) or the empty vector (pET11d), transferred to MOPS agar medium supplemented with 0.4% glucose in the absence (A) or presence (B) of 50 μM IPTG, and incubated for 24 h at 37 °C.

Analysis of OsCRSH Gene Expression—We next investigated the pattern of OsCRSH1 expression in rice plants by Northern hybridization analysis. The expressions of OsSIG1, which encodes plastid RNA polymerase sigma-factor and shows specific expression in green tissues (20), and Rac2, which is constitutively expressed in whole tissues (20), were analyzed as well. A major transcript of 2.1 kb was detected in green tissues and roots, with the amount of this mRNA being greater in the for-mer than in the latter (Fig. 4A). Given that the similarity in the nucleotide sequences of OsCRSH1, OsCRSH2, and OsCRSH3 might result in cross-hybridization of the OsCRSH1 probe in Northern analysis, even under the high stringency conditions
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used, we examined the expression of all three genes by RT-PCR analysis. The abundance of OsCRSH2 and OsCRSH3 mRNAs in both shoots and roots was much lower than that of OsCRSH1 mRNA (Fig. 4B), suggesting that OsCRSH1 is the most highly expressed gene among the three homologs in the rice tissues tested.

**Assay of (p)ppGpp Synthetase Activities of OsCRSH1 in Vitro**—To examine further the enzymatic function of OsCRSH1 as a (p)ppGpp synthetase, we cloned the cDNA for the mature form of the protein into the vector pEGST to yield pGCR1ΔN41. The GST-OsCRSH1(ΔN41) protein encoded by this plasmid was synthesized by *in vitro* transcription and translation and isolated with a glutathione column. The GST portion of the fusion protein was removed on the column by Precision protease, and the GST-OsCRSH1(ΔN41) was further characterized by two-dimensional TLC (Fig. 5B) and confirmed as pppGpp synthetase activity.

The low levels of pppGpp accumulation that were observed in the synthetase reactions under low Ca\(^{2+}\) concentration (Fig. 5A) might be due to the pppGpp hydrolase activity of the OsCRSH1. To assess this possibility, we next examined the hydrolase activity of OsCRSH1. As shown in Fig. 5C, the Mn\(^{2+}\)-dependent pppGpp hydrolase activity was exhibited by *E. coli* SporT, which was tested as a positive control enzyme for pppGpp hydrolase, but the hydrolase activity was not observed in the reactions of OsCRSH1. These results thus indicated that OsCRSH1 possesses Ca\(^{2+}\)-activated pppGpp synthetase activity.

To determine whether the Ca\(^{2+}\) dependence of this enzymatic activity is attributable to the COOH-terminal EF-hand motifs of OsCRSH1, we next constructed single-site mutants in which Ca\(^{2+}\) binding to the individual EF-hands would be expected to be disrupted (27). The mutants OsCRSH1(ΔN41)-EF1 and OsCRSH1(ΔN41)-EF2 thus contain glutamate instead of glutamate at the −Z position of the corresponding Ca\(^{2+}\)-binding loop (Fig. 1C). Neither mutant exhibited Ca\(^{2+}\)-dependent (p)ppGpp synthetase activity (Fig. 5D), indicating that both EF-hand motifs are necessary for this activity.

To examine whether the COOH-terminal region containing the two EF-hand motifs is inhibitory for the pppGpp synthetase activity of OsCRSH1, we prepared a mutant form of the mature protein (OsCRSH1(ΔN41ΔC128)) that lacks the 128 COOH-terminal amino acids (including both EF-hands). The mutant protein did not exhibit (p)ppGpp synthetase activity in the absence or presence of Ca\(^{2+}\) (Fig. 5D), showing that the COOH-terminal region is necessary for enzymatic function.

During preparation of the various forms of OsCRSH1, we noticed that the yield of OsCRSH1(ΔN41) was lower than that of the two EF mutants (data not shown). We therefore monitored the cell-free synthesis of \[^{14}C\]leucine-labeled OsCRSH1(ΔN41) and OsCRSH1(ΔN41)-EF1 in the presence of various concentrations of the Ca\(^{2+}\) chelator 1,2-bis(o-aminophenoxo)ethane-N\(_2\)N\(_2\)N\(_3\)-tetraacetic acid (BAPTA) to deplete endogenous Ca\(^{2+}\). The low yield of OsCRSH1(ΔN41) under the standard condition for cell-free synthesis was increased in the presence of BAPTA (Fig. 5E). In contrast, the high yield of OsCRSH1(ΔN41)-EF1 under the standard condition was not increased further by depletion of Ca\(^{2+}\). Given that stoichiometrically equal amounts of ATP and GTP are converted to pppGpp in the ATP:GTP 5′-pyrophosphotransferase reaction, these results suggested that OsCRSH1(ΔN41) is partially activated in the reaction mixture for cell-free protein synthesis, resulting in depletion of the energy source (ATP and GTP) for protein synthesis and a lower protein yield.

**Translation-coupled (p)ppGpp Synthetase Activity of OsCRSH Proteins**—To examine the enzymatic function of the other two rice CRSH homologs, OsCRSH2 and OsCRSH3, we performed the (p)ppGpp synthetase assay coupled with cell-free protein synthesis. The putative mature forms of the three rice CRSH proteins were thus synthesized and assayed in the presence of \[^{32}P\]ATP and in the absence or presence of BAPTA. Protein synthesis was monitored under identical conditions with the exception that \[^{32}P\]ATP was replaced with \[^{14}C\]leucine. All three rice proteins showed (p)ppGpp synthetase activity in the absence of BAPTA, and the activity of each protein was reduced by depletion of Ca\(^{2+}\) (64). We also observed that three rice CRSH proteins had shown different sensitiv-
ities to BAPTA, suggesting sequence-dependent diversities of their EF-hand functions. Conversely, the synthesis of each protein was increased by the presence of BAPTA (Fig. 6B). These results thus indicated that the three rice CRSH proteins possess similar Ca$^{2+}$-activated (p)ppGpp synthetase activities in vitro.
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**DISCUSSION**

Recent studies have demonstrated the existence of ppGpp in plants (15), the presence of (p)ppGpp synthetase activity in chloroplasts (18), and the nuclear encoding of genes for chloroplastic (p)ppGpp synthetase (16, 17). We have now identified plant genes for a novel type of RelA-SpoT homolog, designated CRSH, that localizes to chloroplasts and shows Ca\(^{2+}\)-dependent (p)ppGpp synthetase activity. The COOH-terminal region of CRSH proteins contains two typical Ca\(^{2+}\)-binding (EF-hand) motifs (28). Expression of the putative mature form of the rice CRSH protein OsCRSH1 in an *E. coli* mutant deficient in RelA and SpoT restored the ability to grow on minimal medium, indicative of functional complementation. Biochemical analysis showed that the (p)ppGpp synthetase activity of rice CRSH proteins is dependent on Ca\(^{2+}\) and that such Ca\(^{2+}\)-dependent activity of OsCRSH1 was abolished by mutation of the Ca\(^{2+}\) binding loop of either EF-hand motif in the COOH-terminal region. Deletion of this COOH-terminal region also abolished the (p)ppGpp synthetase activity of OsCRSH1 in vitro, suggesting not only that the two EF-hand motifs are essential for activation by Ca\(^{2+}\) but that this region of the protein also provides structural support for (p)ppGpp synthetase activity.

With respect to the NH\(_2\)-terminal hydrolase-like domain of the CRSH proteins, their sequences showed no conserved signature corresponding to the feature of the HD domain that is conserved in bacterial hydrolase-active RSH proteins (12). Our experimental results indicated that OsCRSH1 does not have Mn\(^{2+}\)-dependent pppGpp hydrolase activity, and it was consistent with the sequence feature of CRSH proteins.

Evidence indicates that Ca\(^{2+}\) functions as a second messenger in many aspects of plant physiology (29, 30). With regard to identification of the sensors of the cellular Ca\(^{2+}\) signal, gene mining revealed ∼250 genes for EF-hand-containing proteins in the *Arabidopsis* genome (31, 32). These proteins include enzymes, contributors to transcription or translation, participants in protein-protein or protein-nucleic acid interaction, as well as a large number of molecules of unknown function, suggesting that Ca\(^{2+}\)-mediated signaling regulates many processes in plants and that EF-hand-containing proteins are key mediators of Ca\(^{2+}\) action.

In chloroplasts, Ca\(^{2+}\) is required for several important processes. It is thus essential for the function of the oxygen-evolving complex of photosystem II in the thylakoid lumen (33). Stromal Ca\(^{2+}\) also serves a regulatory role in photosynthesis via activation of calmodulin-dependent NAD kinase (34, 35). In addition, trafficking of Ca\(^{2+}\) across the thylakoid membrane regulates the light-dependent activity of enzymes of the Calvin-Benson cycle (36). A Ca\(^{2+}\)-dependent ATPase-like protein and a Ca\(^{2+}\)-H\(^{+}\) antiporter have been identified in the plastid envelope (37) and thylakoid membrane (38), respectively. Furthermore, both circadian oscillation of Ca\(^{2+}\) concentration in chloroplasts (39) as well as a marked increase in stromal Ca\(^{2+}\) concentration after the transition of plants from light to dark (40) have been described. The peak of this latter effect was observed 20–25 min after the transition and represented a change in the free Ca\(^{2+}\) concentration of the stroma from 150 nM to between 5 and 10 μM (40). In the present study, we found that activation of the (p)ppGpp synthetase activity of OsCRSH1 required a Ca\(^{2+}\) concentration of 100 μM in vitro. We previously showed that the (p)ppGpp synthetase activity of intact pea chloroplasts requires the 70 S ribosome (18), suggesting that additional factors such as the 70 S ribosome might increase the activity of OsCRSH1 or render it sensitive to Ca\(^{2+}\) at lower concentrations. An increase in Ca\(^{2+}\) concentration in the chloroplast stroma may thus activate the (p)ppGpp synthetase activity of CRSH proteins and thereby regulate the genetic system of the chloroplast in a manner similar to that apparent in the bacterial stringent response.

The existence and conservation of multiple types of chloroplastic RelA-SpoT homolog, including RSH1 to −3 and CRSH1 to −3, suggest that (p)ppGpp signaling plays an important role in regulation of the genetic system of plastids in land plants. Whereas RSH genes are conserved among bacteria, land plants, and algae, CRSH genes appear to be present only in land plants. We suggest that Ca\(^{2+}\)-activated (p)ppGpp signaling may have arisen together with the evolution of Ca\(^{2+}\) signaling in the chloroplasts of land plants. Such coevolution...
might have enabled land plants to better adapt to adverse circumstances, such as environmental stress, physical injury, and pathogen attack.

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REFERENCES

1. Cashel, M., Gentry, D. R., Hernandez, V. J., and Vinella, D. (1996) Escherichia coli and Salmonella: Cellular and Molecular Biology, 2nd Ed., pp. 1458–1496, ASM Press, Washington, D. C.
2. Magnusson, L. U., Farewell, A., and Nystrom, T. (2005) Trends Microbiol. 13, 236–242
3. Xiao, H., Kalma, M., Ikehara, K., Zemel, S., Glaser, G., and Cashel, M. (1991) J. Biol. Chem. 266, 5980–5990
4. Seyfzadeh, M., Keener, J., and Nomura, M. (1993) Mol. Microbiol. 56, 958–970
5. Hou, Z., Cashel, M., Fromm, H. J., and Honzatko, R. B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11004–11008
6. Vinella, D., Albrecht, C., Cashel, M., and D’Ari, R. (2005) Mol. Microbiol. 56, 939–945
7. Artsimovitch, I., Patlan, V., Sekine, S., Vassilyeva, M. N., Hosaka, T., Ochi, K., Yokoyama, S., and Vassylyev, D. G. (2004) Cell 117, 299–310
8. Milon, P., Tischenko, E., Tomsic, J., Caserta, E., Folkers, G., La Teana, A. L., Rodnina, M. V., Pon, C. L., Boelens, R., and Gualerzi, C. O. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 13962–13967
9. Wang, J. D., Sanders, G. M., and Grossman, A. D. (2007) Cell 128, 865–875
10. Wendrich, T. M., Blaha, G., Wilson, D. N., Marahiel, M. A., and Nierhaus, K. H. (2002) Mol. Cell 10, 779–788
11. Battesti, A., and Bouveret, E. (2006) Mol. Microbiol. 62, 1048–1063
12. Mittenthal, G. (2001) J. Mol. Microbiol. Biotechnol. 3, 585–600
13. Gogfrey, H. P., Burgysrheva, J. V., and Cabello, F. C. (2002) Trends Microbiol. 10, 349–351
14. van der Biezen, E. A., Sun, J., Coleman, M. J., Bibb, M. J., and Jones, J. D. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3747–3752
15. Takahashi, K., Kasai, K., and Ochi, K. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 4320–4324
16. Kasai, K., Usami, S., Yamada, T., Endo, Y., Ochi, K., and Tozawa, Y. (2002) Nucleic Acids Res. 30, 4985–4992
17. Givens, R. M., Lin, M.-H., Taylor, D. J., Mechoild, U., Berry, J. O., and Hernandez, V. J. (2004) J. Biol. Chem. 279, 7495–7504
18. Kasai, K., Kanno, T., Endo, Y., Wakasa, T., and Tozawa, Y. (2004) Nucleic Acids Res. 32, 5732–5741
19. Braeken, K., Moris, M., Daniels, R., Vanderleyden, J., and Michiels, J. (2006) Trends Microbiol. 14, 45–54
20. Tozawa, Y., Tanaka, K., Takahashi, H., and Wakasa, K. (1998) Nucleic Acids Res. 26, 415–419
21. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Sawasaki, T., Ogasawara, T., Morishita, R., and Endo, Y. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14652–14657
23. Madin, K., Sawasaki, T., Ogasawara, T., and Endo, Y. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 559–564
24. Kanno, T., Kitano, M., Kato, R., Omori, A., Endo, Y., and Tozawa, Y. (2007) Protein Expression Purif. 52, 59–65
25. Kanno, T., Komatsu, A., Kasai, K., Dubouzet, J. G., Sakurai, M., Ikejiri-Kanno, Y., Wakasa, K., and Tozawa, Y. (2005) Plant Physiol. 138, 2260–2268
26. Kasai, K., Kanno, T., Akita, M., Ikejiri-Kanno, Y., Wakasa, K., and Tozawa, Y. (2005) Planta 222, 438–447
27. Osawa, M., Dace, A., Tong, K. I., Valiveti, A., Ikura, M., and Ames, J. B. (2005) J. Biol. Chem. 280, 18008–18014
28. Grabarek, Z. (2006) J. Mol. Biol. 359, 509–525
29. Sai, J., and Johnson, C. H. (2002) Plant Cell 14, 1279–1291
30. Reddy, A. S. N. (2001) Plant Sci. 160, 381–404
31. Sanders, D., Pelloux, J., Brownlee, C., and Harper, J. F. (2002) Plant Cell 14, 5401–5417
32. Day, I. S., Reddy, V. S., Ali, G. S., and Reddy, A. S. N. (2002) Genome Biol. 3, 1–24
33. MacCormack, E., Tsai, Y.-C., and Braam, J. (2005) Trends Plant Sci. 10, 384–389
34. Ghanotakis, D. F., Babcock, G. T., and Yocum, C. F. (1984) FEBS Lett. 167, 127–130
35. Jarrett, H. W., Brown, C. J., Black, C. C., and Cormier, M. J. (1982) J. Biol. Chem. 257, 13879–13804
36. Turner, W. L., Waller, J. C., Vanderbeld, B., and Snedden, W. A. (2004) Plant Physiol. 135, 1243–1255
37. Wolosiuk, R. A., Ballicora, M. A., and Hageman, K. (1993) FASEB J. 7, 622–637
38. Huang, L., Berkelman, T., Franklin, A. E., and Hoffman, N. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10066–10070
39. Ettinger, W. F., Clear, A. M., Fanning, K. J., and Peck, M. L. (1999) Plant Physiol. 119, 1379–1385
40. Johnson, C. H., Knight, M. R., Kondo, T., Masson, P., Sedbrook, J., Haley, A., and Trewavas, A. (1995) Science 269, 1863–1865