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Enzymatic and Molecular Characterization of Arabidopsis ppGpp Pyrophosphohydrolase, AtNUDX26

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Not only in bacteria but also in plant cells, guanosine-3′,5′-tetraphosphate (ppGpp) is an important signaling molecule, that affects various cellular processes. In this study, we identified nucleoside diphosphates linked to some moiety X (Nudix) hydrolases, AtNUDX11, 15, 25, and 26, having ppGpp pyrophosphohydrolase activity from Arabidopsis plants. Among these, AtNUDX26 localized in chloroplasts had the highest V_{max} and k_{cat} values, leading to high catalytic efficiency, k_{cat}/K_m. The activity of AtNUDX26 required Mg^{2+} or Mn^{2+} ions as cofactor and was optimal at pH 9.0 and 50 °C. The expression of *AtNUDX26* and of ppGpp metabolism-associated genes was regulated by various types of stress, suggesting that AtNUDX26 regulates cellular ppGpp levels in response to stress and impacts gene expression in chloroplasts. This is the first report on the molecular properties of ppGpp pyrophosphohydrolases in plants.

Key words: Arabidopsis; ganosine 3′,5′-bispyrophosphate (ppGpp); nucleoside diphosphates linked to some moiety X (Nudix) hydrolase

A hyperphosphorylated guanosine nucleotide, ppGpp, termed an alarmone, is a low-molecular-weight effector that helps bacteria survive in limited environments.[1,2] The level of ppGpp in *Escherichia coli* (*E. coli*) increases 10 to 100-fold during amino acid starvation as compared to the level in exponentially growing cells.[3,4] ppGpp also accumulates in cyanobacteria under nitrogen starvation.[5] It binds to the β-subunit of RNA polymerase and induces structural change.[6–10] The ppGpp that accumulates in the cells leads to an increase in the synthesis of many enzymes required for producing amino acids, and to changes in the pattern of expression of various other products.[2,11–14] Notably, it has been reported that the level of ppGpp in plants is increased by environmental stress, including drought, salinity, and UV irradiation.[15] In addition, ppGpp has been found to act as a regulator of chloroplast RNA polymerase.[16]

The biosynthesis of ppGpp in *E. coli* is catalyzed by two homologous enzymes, RelA and SpoT.[2,11] In *E. coli* cells grown under conditions of amino acid depletion, pppGpp is synthesized from ATP and GTP by ribosome-associated RelA, and subsequently converted to ppGpp by 5′-nucleotidase. Thereafter, ppGpp is degraded to GDP and pyrophosphate by SpoT, which has reciprocal activities for ppGpp degradation and synthesis. Van der Biezen *et al.*[10] isolated three *RelA/SpoT* homologs, designated *RSH* genes (*AtRSH1*, *AtRSH2*, and *AtRSH3*), from *Arabidopsis thaliana*. In addition, Arabidopsis had a single *CRSH* gene, a Ca^{2+}-activated *RelA-SpoT* homolog, for an enzyme having ppGpp synthase activity.[17–19] It has been found that ppGpp and the ppGpp synthetase occur in chloroplasts along with all RSH and CRSH proteins in Arabidopsis plants.[15,20–22] The chloroplast is a semi-autonomous organelle that originates not only from an ancestral host cell but also from a cyanobacterium-like photosynthetic prokaryote. Therefore, it is plausible that a stringent control system due to ppGpp similar to that of bacteria exists in plant chloroplasts.[20,23,24]

Nudix hydrolases, distributed in all living organisms, constitute a large family of proteins that share a highly conserved amino acid sequence, $GX_5EX_7REXEEUXGU$, where U is an aliphatic, hydrophobic residue, although several interesting examples exist with altered consensus sequences.[25–27] Nudix hydrolases hydrolyze a wide variety of substrates that contain a nucleoside diphosphate linked to some other moiety, X.[25,27–32] Large numbers of these substrates, including nucleoside di- and tri-phosphates and their oxidized forms, dinucleoside polyphosphates, nucleotide sugars, coenzymes, and capped RNAs, have been identified.[33–37] These substrates are either potentially toxic compounds, cell-signaling molecules, regulators of cellular metabolism, or metabolic intermediates. Hence it has been suggested that Nudix hydrolases play protective, regulatory, and signaling roles in metabolism by hydrolytically removing such compounds.[25,38] *Arabidopsis thaliana* has 28

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Abbreviations: CRSH, Ca^{2+}-activated RelA-SpoT homolog; Nudix, nucleoside diphosphates linked to some moiety X; NUDX, nudix hydrolase; pGp, ganosine 3′,5′-bisphosphate; ppGpp, ganosine 3′,5′-bispyrophosphate; pppGpp, ganosine 3′-diphosphate,5′-triphosphate; RSH, RelA-SpoT homolog

genes (AtNUDX1–27 and AtDCP2) encoding Nudix hydrolase homologs located in the cytosol, mitochondria, and chloroplasts.[28,29,39] There are various AtNUDXs having pyrophosphohydrolase activity, with a wide range of substrate specificities: 8-oxo-7,8-dihydro-2′-(deoxy) guanosine 5′-triphosphate (AtNUDX1), ADP-ribose (AtNUDX2 and 7), ADP-glucose (AtNUDX14), long-chain diadenosine polyphosphates (Ap_nA)(AtNUDX13, 25, 26, and 27), NAD(P)H (AtNUDX6, 7, and 19), coenzyme A (AtNUDX11 and 15), FAD (AtNUDX23), and mRNA caps (AtDCP2).[28,29,39,40] Notably, there is increasing evidence of regulatory roles of the respective Nudix hydrolases in various physiological processes in plants, biotic and abiotic stress responses and the metabolism of various molecules.[39,41–46] However, there are numerous AtNUDXs (AtNUDX3–5, 8, 9, 12, 16–18, 20–22, and 24) that show no activity toward any substrate.

Recently, it was reported that ppGpp is recognized as a substrate for a Nudix hydrolase, Ndx8, in *Thermus thermophilus*,[37] suggesting that Nudix hydrolases have the potential to act as transcriptional regulators through hydrolysis of ppGpp. In fact, intracellular ppGpp levels in the *ndx8* mutant briefly increased at the end of the logarithmic growth phase and then dropped during the stationary phase, indicating that Ndx8 is involved in the regulation of ppGpp levels during growth-phase transition by acting on the degradation of ppGpp.[37] In this study, we identified the ppGpp pyrophosphohydrolases (AtNUDX11, 15, 25, and 26) in Arabidopsis. Among these, judging from its subcellular localization, enzymatic properties (enzymatic ability and kinetic parameters) toward ppGpp, and gene expression in response to environmental stress, AtNUDX26 is involved in the metabolism of ppGpp in chloroplasts.

Materials and Methods

Expression and purification of the recombinant AtNUDX proteins. Recombinant forms of AtNUDXs (AtNUDX1–27) were produced using *E. coli* strain BL21 (DE3) pLysS cells, and were purified from the extract using a HiTrap chelating HP column (GE Healthcare, Little Chalfont, UK) following previous reports.[28,29,40] The protein content was determined by the Bradford method, using bovine serum albumin as standard.[47] The molecular masses of the recombinant AtNUDX proteins agreed with the predicted values, calculated from the amino acid sequence of the mature protein plus the hexahistidine-tag (data not shown).

Enzyme assay and HPLC. ppGpp and ganosine 3′,5′-bisphosphate (pGp) were purchased from TriLink Biotechnologies (San Diego, CA). The hydrolytic activities of recombinant forms of AtNUDXs toward ppGpp were assayed by a method described previously.[28,29] The reaction mixture (60 μL), containing 50 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 25 μM ppGpp, and 0.2–1.0 μg of the purified recombinant protein, was incubated at 37 °C for 10 min. For the assay of K_m, ppGpp at 5 μM to 800 μM was added to the reaction. For the assay of divalent cation-dependency, Mg^{2+} was replaced with Ni^{2+}, Zn^{2+}, Ca^{2+}, or Mn^{2+} (5 mM each). For the assay of pH-dependency, the Tris–HCl buffer was replaced with Glycine-NaOH buffer (100 mM each) at basic pH (pH 8.5–10.0). Temperature dependency was assayed at 20–60 °C. The reaction was terminated by adding 10 μL of 100 mM EDTA. In addition, the reaction was carried out under reducing conditions with 1 mM DTT following Olejnik *et al.*[48] The mixture was then analyzed by HPLC using a COSMOSIL C18 column (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan) at a flow rate of 0.6 mL/min for the mobile phase buffer, which contained 73 mM KH_2PO_4, 5 mM tetrabutylammonium dihydrogenphosphate, and 21% methanol. The substrates (ppGpp) and the reaction product (pGp) were detected by their UV absorbances at 252 nm.

Plant materials and stress treatments. Arabidopsis thaliana wild-type (Col-0) was grown on Murashige and Skoog's medium under a light intensity of 100 μmol photons/m^2/s. Two-week-old Arabidopsis plants were subjected to various types of stress: salinity, paraquat, drought, and heat. Paraquat at 50 μM was sprayed on the plants. Salinity stress was imposed by transferring the plants to MS medium containing 250 mM NaCl for 0 to 9 h. Drought stress was imposed by subjecting them to dehydration on paper towels for 0 to 6 h. Heat stress was imposed by incubating the cultures for 0 to 3 h at 37 °C.

Quantitative Real-Time PCR experiments. Quantitative Real-Time PCR (RT–PCR) experiments were performed following Nishizawa *et al.*[49] The primer sequences were as follows:
NUDX26-primerF (5′-ATCCGATTGGAAGGGTCAAGCAC-3′),
NUDX26-primerR (5′-CTCAGGTTTCTCAGTACCATCGC-3′),
RSH1-primerF (5′-TTCTTGCGTGGCCGAGATTGACA-3′),
RSH1-primerR (5′-CACCTTAGCGCAAACACACTAACCAAACT-3′),
RSH2-primerF (5′-CTGAAGATGGGTGATGTGGTGG-3′),
RSH2-primerR (5′-CGCGAGTCCTCGATCATACATG-3′),
RSH3-primerF (5′-ACACCTGTAAGTGATCTGAAATGCAAG-3′),
RSH3-primerR (5′-CGCAAGCCCTCGATCATACATC-3′),
CRSH-primerF (5′-CAGACGAATTCGATACGTTTCAGAAAC-3′),
CRSH-primerR (5′-TCATGAAGCTTCTCGTCTAAAAGACTC-3′),
Actin2-QF (5′-GGTGGTTCCATTCTTGCTTCCC-3′),
Actin2-QR (5′-TCATACTCGGCCTTGGAGATCC-3′).

Data analysis. Significance of differences between data sets was evaluated by *t*-test. Calculations were carried out with Microsoft Excel software.

Accession numbers. The Arabidopsis Genome Initiative locus identifiers for the genes mentioned here are as follows: *AtNUDX1* (At1g68760), *AtNUDX2* (At5g47650), *AtNUDX3* (At1g79690), *AtNUDX4* (At1g18300), *AtNUDX5* (At2g04430), *AtNUDX6* (At2g04450), *AtNUDX7* (At4g12720), *AtNUDX8* (At5g47240), *AtNUDX9* (At3g46200), *AtNUDX10* (At4g25434), *AtNUDX11* (At5g45940), *AtNUDX12* (At1g12880), *AtNUDX13* (At3g26690), *AtNUDX14* (At4g11980), *AtNUDX15* (At1g28960), *AtNUDX16* (At3g12600), *AtNUDX17* (At2g01670), *AtNUDX18* (At1g14860), *AtNUDX19* (At5g20070), *AtNUDX20* (At5g19460), *AtNUDX21* (At1g73540), *AtNUDX22* (At2g33980), *AtNUDX23* (At2g42070), *AtNUDX24* (At5g19470), *AtNUDX25* (At1g30110), *AtNUDX26* (At3g10620), *AtNUDX27* (At5g06340), *RSH1* (At4g02260), *RSH2* (At3g14050), *RSH3* (At1g54130), *CRSH* (At3g17470), and *Actin2* (At3g18780).

Results and Discussion

Identification of AtNUDXs having pyrophosphohydrolase activity toward ppGpp

To identify AtNUDXs having pyrophosphohydrolase activity toward ppGpp, we prepared His-tagged recombinant AtNUDX1–27 proteins in the absence of the predicted transit peptide expressed in *E. coli* cells. Production and purification of the recombinant AtNUDXs were carried out by a method reported previously.[28,29,40] The hydrolase activity of AtNUDXs toward ppGpp in the presence of 5 mM Mg^{2+} as cofactor was examined by HPLC analysis. Recombinant AtNUDX26 hydrolyzed ppGpp with relatively high activity (0.19 ± 0.05 μmol/min/mg protein). AtNUDX11 (0.06 ± 0.01 μmol/min/mg protein), 15 (0.02 ± 0.01 μmol/min/mg protein), and 25 (0.06 ± 0.01 μmol/min/mg protein) exhibited barely detectable activity. No ppGpp hydrolase activity was detected in the other AtNUDXs (AtNUDX1–10, 12–14, 16–24, and 27).

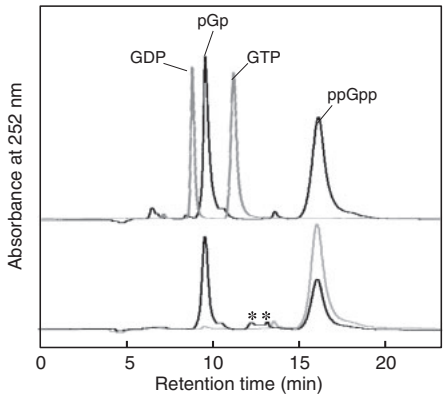

Fig. 1. Identification of the Products of the Degradation of ppGpp by AtNUDX26.

Reaction mixtures containing 25 μM (1.5 nmol/60 μL) ppGpp were incubated with 5 mM Mg^{2+} in the absence and the presence of the purified recombinant AtNUDX26 protein at 37 °C for 10 min and then subjected to HPLC with a COSMOSIL C18 column, as described in "Materials and Methods." The elution profiles of the reaction mixture without the enzyme (gray line) and with the enzyme (black line) is shown. Positions of standards (0.9 nmol of GDP, 1.5 nmol of GTP, 1.5 nmol of ppGpp, and 1.5 nmol of pGp) are shown in the upper panel. Asterisks indicate either pGpp or ppGp.

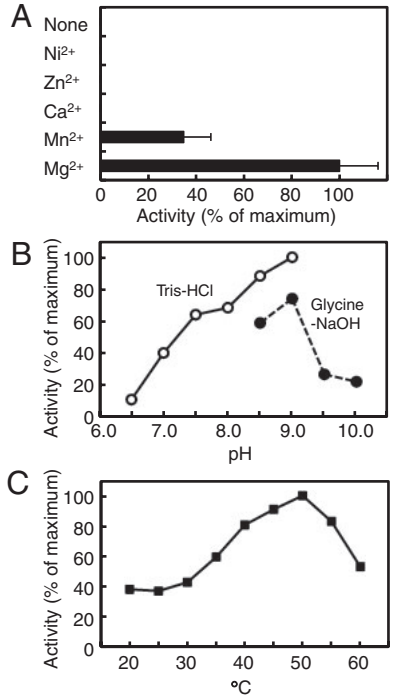

Fig. 2. Enzymatic Characterization of AtNUDX26.

A, The requirement of divalent cations for ppGpp pyrophosphohydrolase activity was determined in the presence and the absence of divalent cations (5 mM) at 37 °C for 10 min, as described in "Materials and Methods." B, The pH-dependency of ppGpp pyrophosphohydrolase activity was determined under the assay conditions described in "Materials and Methods," except that Tris–HCl buffer was replaced with Glycine-NaOH buffer (100 mM each) at the indicated pH. C, The temperature dependency of ppGpp pyrophosphohydrolase activity was determined as described in "Materials and Methods," at 20–60 °C.

It has been reported that the activity of some Nudix hydrolases, such as AtNUDX13 and 26, which have Ap_nA pyrophosphohydrolase activity, increased under reducing conditions.[29,48] However, in the presence of the reducing agent, dithiothreitol (DTT: 1 mM), in the reaction solution, the activities of AtNUDX11 (0.05 ± 0.02 μmol/min/mg protein), 25 (0.05 ± 0.03 μmol/min/mg protein), and 26 (0.12 ± 0.04 μmol/min/mg protein) were similar to those in the absence of the agent. No ppGpp hydrolase activity was detected for the other AtNUDXs under reducing conditions (data not shown).

The reaction products generated by the activity of AtNUDX26 toward ppGpp were determined by HPLC analysis (Fig. 1). SpoT and its homolog, RSH, degrade ppGpp to GDP and pyrophosphate.[2,10,11] Typical Nudix hydrolase cleaves the 5′-pyrophosphate linkage of its substrate. On the other hand, *Thermus thermophilus* Ndx8 degrades ppGpp to pGp and two phosphates by hydrolysis not only of the 5′-diphosphate linkage but also of 3′-diphosphate linkage of ppGpp.[37] 3′-Diphosphatase activity is an unique property of Ndx8. In addition to the peak of ppGpp as substrate, three peaks with absorbance at 252 nm were detected in the reaction solution. The peak that eluted first (approximately 9.50 min) corresponded to that of standard pGp, while the others were estimated to be either pGpp or ppGp. These findings indicate that like *Thermus thermophilus* Ndx8, AtNUDX26 has pyrophosphohydrolase activity toward both diphosphate linkages in ppGpp, and generates pGp as final product. At present, the fates of pGp and pGpp/ppGp are unknown, even in bacterial cells.[37]

Enzymatic properties and kinetic parameters of AtNUDX26 as a ppGpp pyrophosphohydrolase

Most previously characterized Nudix hydrolases require bivalent metal ions for their activity. Hence we studied the effects of a series of bivalent metal ions on the activity of AtNUDX26 (Fig. 2A). The activity of AtNUDX26 was detected only in the presence of Mg^{2+} and Mn^{2+}. No activity was detected in the absence of these metal ions, or in the presence of the other metal ions tested.

Table 1. Analysis of the Enzymatic Properties of AtNUDX11, 15, 25, and 26

The standard assay was used with concentrations of 5 to 800 μM for ppGpp at 37 °C with 5 mM Mg^{2+}, as described in "Materials and Methods." Data are means of three independent determinations ± SD.

Protein	K_m μM	V_{max} μmol/min/mg	k_{cat} 1/s	k_{cat}/K_m 1/s/M
AtNUDX11	29.1 ± 2.5	0.15 ± 0.03	0.07	2.3×10^3
AtNUDX15	145.3 ± 9.8	0.05 ± 0.01	0.03	2.0×10^2
AtNUDX25	63.7 ± 3.6	0.35 ± 0.07	0.12	1.9×10^3
AtNUDX26	135.1 ± 9.6	1.64 ± 0.30	0.49	3.6×10^3

The optimum pH for activity was determined using Tris–HCl (pH 6.5–9.0) and glycine–NaOH (pH 8.5–10.0) buffers. As shown in Fig. 2B, AtNUDX26 was most active at pH 9.0, with 50% activity at pH 7.0 and pH 9.5. The optimum temperature for the enzyme was 50 °C (Fig. 2C).

Kinetic parameters for ppGpp were measured (Table 1). The K_m values of AtNUDX11, 15, 25, and 26 were 29.1 ± 2.5, 145.3 ± 9.8, 63.7 ± 3.6, and 135.1 ± 9.6 μM respectively. The V_{max} of AtNUDX26 was 1.64 ± 0.30 μmol/min/mg, the highest value obtained. Thus AtNUDX26 had the highest catalytic efficiency (k_{cat}/K_m) for ppGpp among these AtNUDXs. It has been reported that AtNUDX26, with a chloro-

plastic transit peptide, is distributed in the chloroplasts of Arabidopsis cells, whereas AtNUDX11 and 25 are in the cytosol and AtNUDX15 is in the mitocondria.[28,29] In addition, AtNUDX11 and 15 have higher hydrolysis activity toward CoA and its derivatives than ppGpp, and thus appear to act on the regulation of various CoA-related metabolisms.[39] On the other hand, AtNUDX25 and 26 have highest activity toward Ap_nA among various substrates,[40] but the substantial physiological role of Ap_nA remains unclear. These findings and the results obtained here suggest that AtNUDX26 contributes to the regulation of ppGpp levels *via* hydrolysis in the chloroplasts.

Comparison of amino acid sequences between AtNUDXs and T. thermophilus *Ndx8*

Nudix hydrolases contain the highly conserved Nudix box $GX_5EX_7REXEEUXGU$, where U is a bulky hydrophobic amino acid such as Ile, Leu, or Val.[25–27] In addition, some Nudix hydrolases, with hydrolytic activity toward ADP-ribose (Proline 15 or 16 aa downstream of the Nudix box), Ap_nA ($GGGX_5EX_7REUXEEXGUX_2GX_6G$), coenzyme A (UPF0035; $LLTXR[SA]X_3RX_3GX_3FPGG$), NADH (SQPWPFPQS), or UTP (L[VL]VRK and AANE), contain additional regions conserved across several species (PROSITE: http://prosite.expasy.org/).[27,31,39,50–53] Previous studies have found that AtNUDX11 and 15 have the UPF0035 motif conserved in CoA pyrophosphohydrolases.[39] AtNUDX25 and 26 have been identified as Ap_nA pyrophosphohydrolases with a unique conserved amino acid sequence ($GGGX_5EX_7REUXEEXGUX_2GX_6G$).[29,31,40] This suggests that the mechanism of the recognition of similar to that CoA and Ap_nA.

Next, we compared the amino acid sequences of AtNUDXs with *T. thermophilus* Ndx8, known to be a ppGpp pyrophosphohydrolase.[37] Ndx8 did not share significant sequence homology to any of the AtNUDXs (3–24%), including AtNUDX11 (19%), 15 (17%), 25 (24%), and 26 (18%), and no region other than the Nudix motif was conserved among them (data not shown). These findings suggest that plant Nudix hydrolases having ppGpp pyrophosphohydrolase activity undergo their own evolutionary process. Alternatively, there might be unidentified amino acid residues essential for ppGpp pyrophosphohydrolase activity, since several residues have been reported to be essential for activity or substrate recognition in certain NUDXs, including AtNUDX1 and *E. coli* NudG.[31]

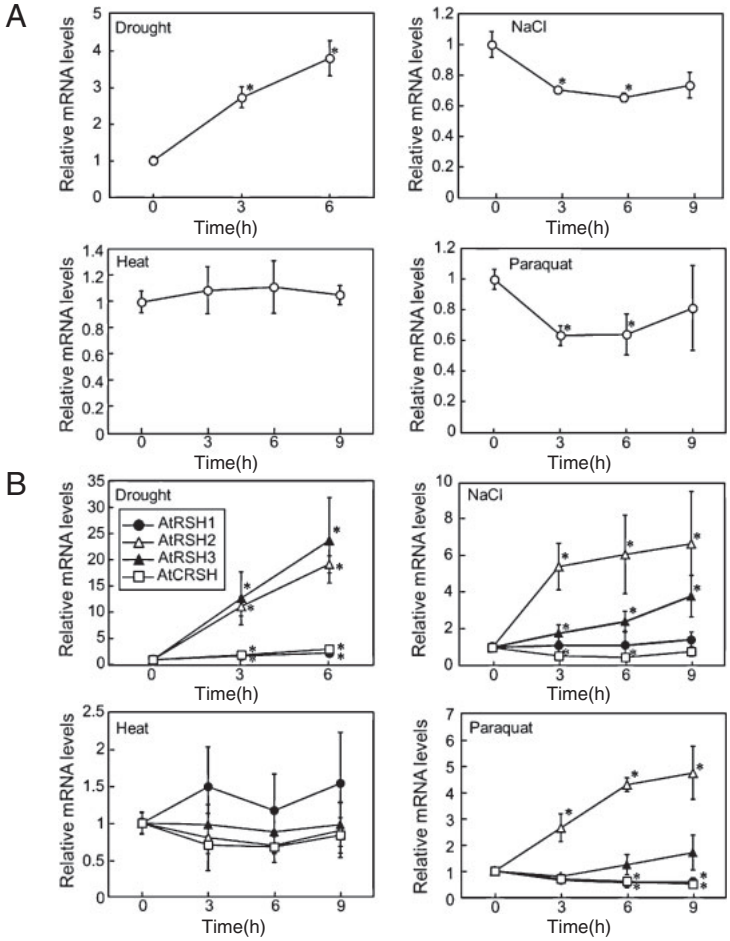

Fig. 3. Expression Profiles of *AtNUDX26*, *AtRSH1*, *AtRSH2*, *AtRSH3*, and *CRSH* under Various Stressful Conditions.

Two-week-old Arabidopsis plants were subjected to various forms of stress: treatment with salinity (250 mM NaCl), PQ (50 μM), drought (dehydration on a paper towel), and heat (37 °C under dark conditions). Total RNA extracted from Arabidopsis leaves was converted into first-strand cDNA with the oligo $(dt)_{20}$ primer. A quantitative PCR analysis was carried out to determine the expression levels of AtNUDX26 (A) and *AtRSH1*, *AtRSH2*, *AtRSH3*, and *CRSH* (B). The details of the procedures are described in "Materials and Methods." Relative amounts were normalized to *Actin2* mRNA. Data are mean values ± SD for three individual experiments ($n = 3$). Asterisks indicate values significantly different from the control treatment ($p < 0.05$).

Expression of AtNUDX26 in response to various types of stress

It has been reported that ppGpp levels in plant cells increased in response to various types of environmental stress.[15] We analyzed changes in the expression of *AtNUDX26* and the genes encoding enzymes involved in ppGpp metabolism, *AtRSH1*, *AtRSH2*, *AtRSH3*, and *CRSH*, under various stressful conditions. RT–PCR analysis revealed that the transcript levels of *AtNUDX26* increased approximately 4-fold at 6 h after drought stress (Fig. 3). A significant increase in expression under drought stress was observed for all the genes tested here. In particular, the levels of *AtRSH2* and *AtRSH3* increased approximately 19- and 24-fold respectively. Conversely, expression of *AtNUDX26* decreased under oxidative stress caused by treatment with paraquat (PQ, a generator of reactive oxygen species) and under salinity caused by treatment with NaCl (Fig. 3). Similarly, the levels of *CRSH* decreased in response to both PQ and NaCl treatments. In contrast, the levels of *AtRSH2* increased in response to these treatments. The expression of *AtNUDX26* was not significantly changed by treatment with heat, although the expression levels of some *AtRSHs* were affected (Fig. 3). These results suggest that the metabolism of ppGpp is regulated depending on the type of stress, and that AtNUDX26 might contribute to regulation.

Conclusion

In plant cells, ppGpp is an important signaling molecule, that affects various cellular processes, including transcription, translation, DNA replication, amino acid and nucleotide metabolism, secondary metabolism, and infectivity.[2,11–14,54] For the first time, we characterized the molecular and enzymatic properties of ppGpp pyrophosphohydrolases in plants, AtNUDX11, 15, 25, and 26. It has been found that in plant chloroplasts ppGpp is synthesized and degraded by RSHs and directly affects the translation system.[15,20–22,54,55] In addition to the importance of ppGpp metabolism in plant chloroplasts, the finding that among AtNUDXs having ppGpp pyrophosphohydrolase activity, chloroplastic AtNUDX26 has the highest catalytic efficiency and that its expression is regulated in response to various types of environmental stress suggests that AtNUDX26 impacts the metabolism of ppGpp in chloroplasts through the hydrolysis of ppGpp under stress. Since ppGpp is a substrate for the hydrolysis reaction by AtNUDX26, it might play a role in the fine tuning of ppGpp signaling in combination with AtRSHs in the chloroplasts. In addition, it is possible that not only ppGpp but also pGp, the final product of the reaction of AtNUDX26, have signaling roles in cellular responses, since its analog, 3′-phosphoadenosine 5′-phosphate, has an inhibitory effect on certain enzymes, such as 3′-phosphoadenosine 5′-phosphosulfate, nucleoside diphosphate kinase, and poly (ADP-ribose) polymerase, which are involved in various biological processes.[56–61] To determine the importance of the degradation of ppGpp by AtNUDX26, we are analyzing the effect of the knockout and overexpression of *AtNUDX26* on levels of ppGpp and the response to various types of stress.

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