Comprehensive analysis of Cytosolic Nudix hydrolases in *Arabidopsis thaliana*
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Nudix hydrolases are a family of proteins that catalyze the hydrolysis of a variety of nucleoside diphosphate derivatives. Twenty-four genes of the Nudix hydrolase homologues (AtNUDTs) with predicted localizations in the cytosol, chloroplasts and mitochondria exist in *Arabidopsis thaliana*. Here, we demonstrated the comprehensive analysis of 9 types of cytosolic AtNUDT proteins (AtNUDT1, 2, 4, 5, 6, 7, 9, 10 and 11). The recombinant proteins of AtNUDT2, 6, 7 and 10 showed both ADP-ribose and NADH pyrophosphatase activities with significantly high affinities compared with those of animal and yeast enzymes. The expression of each AtNUDT is individually regulated in different tissues. These findings suggest that most cytosolic AtNUDTs may substantially function in the sanitization of potentially hazardous ADP-ribose and the regulation of the cellular NADH/NAD⁺ ratio in plant cells. On the other hand, the AtNUDT1 protein had the ability to hydrolyze 8-oxo-dGTP with a *Km* value of 6.8 μM and completely suppress the increased frequency of spontaneous mutations in the *E. coli mutT* strain, indicating that AtNUDT1 is a functional homologue of *E. coli* MutT in *A. thaliana* and is involved in the prevention of spontaneous mutation. The results obtained here suggest that the plant Nudix family has evolved in a specific manner that differs from that of yeast and humans.

Nudix (nucleoside diphosphates linked to some moiety X) hydrolases that are characterized by a conserved Nudix motif: GXᵦEXᵦREVXEEGUX, where *X* is usually Ile, Leu or Val (1), are widely distributed in over 120 species, ranging from viruses to humans (2). It has been proposed that Nudix hydrolases may be divided into subfamilies based on their major substrates: dinucleoside polyphosphates, ADP-ribose, NADH, nucleotide sugars, or ribo- and deoxy-nucleoside triphosphates (3). Since the accumulations of their substrates are often toxic to the cell, their intracellular levels need to be precisely regulated by these enzymes (1).

Among the Nudix hydrolases, *E. coli* MutT protein hydrolyzes all canonical nucleoside triphosphates with a preference for 8-oxo-dGTP (8-oxo-2'-deoxyguanosine-5'-triphosphate) and 8-oxo-GTP (8-oxo-2'-guanosine-5'-triphosphate), the oxidized form of the free guanine nucleotide, by attacking an active oxygen species, such as a superoxide radical (O₂₋), H₂O₂ or a hydroxyl radical (·OH), to the monophosphate form. Since 8-oxo-G (8-oxo-7, 8-dihydroguanine) can be incorporated into the nascent strand opposite the adenine and cytosine in the template with almost equal efficiency, resulting in an A:T to C:G transversion mutation (4,5), MutT functions in the prevention of the misincorporation of such mutagenic nucleotides into DNA or mRNA during DNA replication or transcription (5,6). In human cells, the accumulation of 8-oxo-G may be...
responsible for a significant portion of spontaneous mutations that lead to the induction of cancer, as well as age-related disorders (7). Homologues of *E. coli* MutT in human (hMTH1) and rodent cells were identified, and they complement the function of MutT in *E. coli* (8-11).

In contrast to human and *E. coli*, little is known about the functions of Nudix hydrolases and defense systems toward oxidative damage of nucleotides by MutT proteins in higher plants (12). There are important differences between the life strategies of plants and most eukaryotes. Especially, even under optimal conditions, the photosynthetic electron transport in chloroplasts is inevitably accompanied by the reduction of O₂ to O₂⁻, which is followed by the production of other active oxygen species such as H₂O₂ and **OH, on the reducing side of Photosystem I, although light, as the energy source for photosynthesis, is essential for plant life (13,14). Furthermore, a wide range of environmental stresses, such as drought, high salinity, and low temperature result in the enhanced production of active oxygen species (14-18). Based on a BLAST search (19) of the DNA database, it is likely that 24 open reading frames encoding potential homologues of Nudix hydrolases exist in *Arabidopsis thaliana*. Among them, AtNUDT1 (At1g68760) was initially characterized as a NADH pyrophosphatase (20). In addition, Klaus *et al.* (2005) recently reported that AtNUDT1 was the closest homologue of *Lactococcus lactis* YlgG and showed activities toward both dihydrodopterin triphosphate, a precursor of folate, and (deoxy) nucleoside triphosphates including 8-oxo-dGTP (21). However, little attention has been given to the effects of MutT-type proteins, including AtNUDT1, on the sanitization system of oxidized nucleotides.

Furthermore, several members of the Nudix hydrolase family have recently been described as having either a high specificity for ADP-ribose or including ADP-ribose within their substrate specificity range (3). Although it is yet to be proven, it is assumed that these enzymes play an important role in maintaining the concentration of intracellular ADP-ribose at a sub-toxic level, since the accumulation of ADP-ribose can be potentially cytotoxic due to its ability to modify protein and to bind to ATP-activated K⁺ channels (22). It has been demonstrated that the gene products from *Methanococcus jannaschii* MJ1149, *E. coli* orf186 and *Saccharomyces cerevisiae* YSA1, and the human and mouse Ysa1p homologues, NUDT5 and Nudt5, respectively, have activities towards ADP-ribose (3, 23-25).

In this work, we characterized the molecular properties of cytosolic Nudix hydrolases in *A. thaliana*, since the cytosol is the main pool of their substrates. For the first time, we demonstrated that AtNUDT1 functions in the defense system of spontaneous mutagenesis via the sanitization of oxidized nucleotides. Furthermore, enzymatic characterizations of a large number of ADP-ribose pyrophosphatases in *A. thaliana* clearly suggest that these isoenzymes play roles in regulating the intracellular concentration of ADP-ribose in plant cells.

**EXPERIMENTAL PROCEDURES**

**Materials and plant growth conditions---** *Arabidopsis thaliana* (ecotype Columbia) plants were grown on basic Murashige and Skoog medium in petri dishes containing 3% (W/V) sucrose at 25°C under long-day conditions (16 h light at 100 µEm⁻²/s⁻¹ / 8 h dark period).

The *E. coli* strain, CC101, and the *mutT* deficient strain, CC101T, were kind gifts from Prof. Maki (NARA INSTITUTE of SCIENCE and TECHNOLOGY). The pTrc100 plasmid for the complementary assay was obtained from Prof. Nakabeppu (Kyushu University). The 8-oxo-dGTP was purchased from TriLink Biotechnologies (San Diego, USA). Restriction enzymes and modifying enzymes were purchased from TaKaRa (Kyoto, Japan). All other materials and enzymes were of analytical grade and were obtained from commercial sources.

**Construction of expression plasmids of**
recombinant AtNUDT---Total RNA was isolated from the leaves of 4-week-old wild-type plants (1.0 g fresh weight), as previously described (26). The first strand cDNA was synthesized using ReverTra Ace (reverse transcriptase; Toyobo) with an oligo dT primer according to the manufacturer’s instructions. The open reading frames of AtNUDT1~11 were amplified from the first strand cDNAs, using the primer sets shown as follows; AtNUDT1-Nde I-F (5‘-CATATGTCCAGACGAGAAACG-3’), AtNUDT1-Nde I-R (5‘-CATATGTTAACTCTTACATC-3’), AtNUDT2-Nde I-F (5‘-CATATGTTAACTCTTACATC-3’), AtNUDT2-Nde I-R (5‘-CATATGGACGGTGTTTCTCTT-3’), AtNUDT4-Nde I-F (5‘-CATATGGACGGTGTTTCTCTT-3’), AtNUDT4-BamH I-R (5‘-GGATCCAGAGGGTCTCTGT-3’), AtNUDT5-Nde I-F (5‘-CATATGACATATGCTTCTATC-3’), AtNUDT5-Nde I-R (5‘-CATATGACATATGCTTCTATC-3’), AtNUDT6-Nde I-F (5‘-CATATGGACGAATGAGATCA-3’), AtNUDT6-Nde I-R (5‘-CATATGCACGTTCTGAGAA-3’), AtNUDT7-Nde I-F (5‘-CATATGGTACTAGCTAGAC-3’), AtNUDT7-Nde I-R (5‘-CATATGTCGGAATAATATAG-3’), AtNUDT8-Nde I-F (5‘-CATATGGTATTCTGTTTCTCT-3’), AtNUDT8-Nde I-R (5‘-CATATGGAAAGAGAGATCG-3’), AtNUDT9-Nde I-F (5‘-CATATGGCAAACTGCAAGAAGAT-3’), AtNUDT9-BamH I-R (5‘-GGATCCAGACGAGAAACG-3’), AtNUDT10-Nde I-F (5‘-CATATGGCAGACAGAGCTTCCAT-3’), AtNUDT10-BamH I-R (5‘-GGATCCAGACGAGAAACG-3’), AtNUDT11-BamH I-R (5‘-GGATCCAGACGAGAAACG-3’), AtNUDT11-BamH I-R (5‘-GGATCCAGACGAGAAACG-3’). The sequences were homologous to the cDNAs of AtNUDT1~11 except for the replacement of the original nucleotides that introduced the desired restriction sites (bold sequences). The amplified DNA fragments were ligated into pSTBlue T-vectors (Novagen). DNA sequencing was performed using the dideoxy chain terminator method with an automatic DNA sequencer (ABI PRISM™ 310, Applied Biosystems). The resulting constructs were digested with Nde I/Nde I for AtNUDT 1, 2, 4, 5, 6, 7 and 8, Nde I/BamH I for AtNUDT9 and 10, and Xho I/Xho I for AtNUDT11, and were ligated into the expression vectors, pET16b (Novegen) or pCold II (TaKaRa), to produce Histidine-tagged proteins. The resulting plasmids, pET16b/AtNUDT 1, 2, 4, 5, 6, 7 and 8, and pCold II/AtNUDT 4, 8, 9, 10 and 11 were introduced into E. coli strain BL21 (DE3) pLysS cells.

Expression and purification of recombinant AtNUDT proteins---E. coli strain BL21 (DE3) pLysS transformed with pET16b/AtNUDT 1, 2, 4, 5, 6, 7 or 8, or pCold II/AtNUDT 4, 8, 9, 10 or 11 was grown in 50 ml of LB medium containing 50 µg ml⁻¹ of ampicillin and 34 µg ml⁻¹ of chloramphenicol. After an overnight culture at 37°C, the cultures were transferred to 500 ml of LB medium (with the antibiotics) and grown to an A₆₀₀ of 0.6. Isopropyl-1-thio- β-D-galactopyranoside was added to a concentration of 0.4 mM and the cells were incubated for 16 h at 16°C. The harvested cells were resuspended in Tris-HCl (pH 8.0) containing 0.5 M NaCl, 5 mM imidazole and 1 mM 2-mercaptoethanol, and were sonicated (10 kHz) using 20-s strokes with 30-s intervals and were centrifuged at 15,000 x g for 15 min. The hexahistidine-tagged recombinant AtNUDT proteins were purified from the soluble fraction using a HiTrap chelating HP column (Amersham Biosciences) according to the manufacturer’s instructions. Protein contents were determined following the method of Bradford (27).

Enzyme assay and HPLC analysis---The hydrolytic activities of AtNUDT proteins towards 8-oxo-dGTP, ADP-ribose and
NADH were assayed according to the method described in Tassotto and Mathews (28) with some modifications. Sixty microliters of the reaction mixture, containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 25 μM substrate and 0.2–10 μg of recombinant proteins was incubated at 37°C for 10 min. The reaction was terminated by adding 10 μl of 100 mM EDTA. The mixture was then analyzed with HPLC using a COSMOSIL C₁₈ column (4.6 X 250 mm, Nacalai tesque) at a flow rate of 0.6 ml min⁻¹ for the mobile phase buffer, which contained 73 mM KH₂PO₄, 5 mM tetrabutylammonium dihydrogenphosphate, and 20 % methanol. The substrates and their reaction products were detected according to their UV absorbance, as follows; 8-oxo-dGTP: 293 nm, dGTP: 252 nm, dCTP: 271 nm, dTTP: 264 nm, ADP-ribose, NADH, FAD, CoA, ApnA, UDP-glucose, and UDP-galactose: 260 nm.

Complementation assay of the E. coli mutT mutation---AtNUDT cDNAs were amplified with the RT-PCR using the specific primer sets as shown; AtNUDT1-NcoI-F (5'-CATGCGACAGGAGAAGCG-3'), AtNUDT1-KpnI-R (5'-GGATCCCTCTCTCCTCCACCCAG-3'), AtNUDT2-SacI-F (5'-AGATCTCTCTGTTTTG-3'), AtNUDT2-EcoRI-F (5'-GAATTCGTTCTAGACTGACA-3'), AtNUDT2-SacI-R (5'-GATCCCGAGCTTCTG-3').

The amplified DNA fragments were digested with Nco I/Kpn I (AtNUDT1), Sac I/Xba I (AtNUDT2) and Eco RI/Bam HI (AtNUDT7), respectively, and were ligated into pTrc100 vectors.

The complementation assay was carried out according to the method described in Sakumi et al. (8). E. coli strains CC101 (wild type) or CC101T (mutT) were transformed with empty plasmids, or the plasmids containing AtNUDT cDNAs. A single transformant was grown in 5 ml of LB medium containing 100 μg ml⁻¹ ampicillin at 37°C overnight. The culture solutions were diluted 1 x 10⁶ fold and 100 μl of each culture was grown in 6 ml of LB medium containing 1 mM IPTG for 14~16 hours. Mutation frequencies toward streptomycin resistance were measured by plating aliquots of these cultures on LB medium with or without the antibiotic (100 μg ml⁻¹).

Analysis of AtNUDT expression---Total RNAs were isolated from several tissues (roots, stems and leaves) of 2-week-old wild-type A. thaliana plants (1.0 g fresh weight). The cDNAs encoding AtNUDT proteins were amplified with the RT-PCR (20–28 cycles) using the following primer sets;

AtNUDT1-F (5'-ATAATGTCGACAGGAAGAGC-3'), AtNUDT1-R (5'-CATCTAATGACAGGAGACTT-3'), AtNUDT2-F (5'-TTCACTGTGCTCCACCCAGC-3'), AtNUDT2-R (5'-AGATCTCTGAGGAGACTC-3'), AtNUDT3-F (5'-CATATGGCGGAGGAGCACTT-3'), AtNUDT3-R (5'-GGATCCGCTGAAGACTAAC-3'), AtNUDT4-F (5'-CATATGGAGGTTCCTGTC-3'), AtNUDT4-R (5'-GGATCCAGTGACTAATC-3'), AtNUDT5-F (5'-TGATACGAGGACTGAGGCACG-3'), AtNUDT5-R (5'-CTAGTCAGAGGAGAGGCTG-3'), AtNUDT6-F (5'-TGAAATAGGGTGGGCTAG-3'), AtNUDT6-R (5'-CAACCAGATTGGCTAGG-3'), AtNUDT7-F (5'-TGAGATGGTACATGAGCTC-3'), AtNUDT7-R (5'-CAGAGAGGAGAGGCTG-3'), AtNUDT8-F (5'-CCACATAGATCTTCTTGGATC-3'), AtNUDT8-R (5'-GAGGATGAGGAGGATAC-3'), AtNUDT9-F (5'-CATATGGGAAACTAGCAGAGAGGTTGAT-3'), AtNUDT9-R (5'-GGATCCCAATAATTCACACTAGG-3'), AtNUDT10-F (5'-CATATGTCAGACCAAGAGGCTCCCT-3').
RESULTS

Nudiv hydrolase genes in A. thaliana---A search for the Nudiv hydrolase genes in the National Center for Biotechnology Information Data Base (http://www.ncbi.nlm.nih.gov/) showed that 24 genes (AtNUDT1~24) encoding the Nudiv domain existed in A. thaliana. The portions of the open reading frames of 24 genes are characterized by a signature sequence of highly conserved amino acids spanning a region of 23 amino acids called the Nudiv box, which acts as the catalytic and nucleotide binding sites (Fig. 1). Among them, only AtNUDT1 has been characterized as an NADH, dihydronperin triphosphate, and (deoxy) nucleoside triphosphate pyrophosphatases (20, 21). The deduced amino acid sequences of AtNUDTs showed low homology (11.6~25.9%) with those of E. coli MutT and human MTH1, and their sizes ranged from 16.4 KDa (147 amino acids) to 86.9 KDa (772 amino acids). Furthermore, it was predicted that these AtNUDTs were classified into 3 types by their subcellular localization in the TargetP (http://www.cbs.dtu.dk/services/TargetP/) as follows; cytosol (AtNUDT1~11), mitochondrion (AtNUDT12~18) and chloroplast (AtNUDT19~24). Among them, the product of AtNUDT3 is not likely a typical Nudiv hydrolase enzyme, since its predicted sequence shows an extremely low homology to those of other AtNUDTs, and its molecular weight (86.9 kDa) is much higher than those of the enzymes in other organisms, such as E. coli and human. In eukaryotic cells, pools of their substrates are present mainly in the cytosol (29), and human MTH1 is mostly localized in the cytosolic fraction (30). Therefore, we focused on cytosolic types of AtNUDTs (AtNUDT1, 2 and 4~11) and studied their molecular properties.

Expression and purification of the His-tagged recombinant AtNUDT proteins---The cDNAs encoding cytosolic AtNUDTs were obtained from total RNA prepared from rosetta leaves of A. thaliana. The entire ORF regions of AtNUDT1, 2 and 4~11 were cloned into pET16b or pCold II for production of their recombinant proteins fused with a hexahistidine-tag in E. coli. The recombinant proteins of AtNUDT1, 2, 4, 5, 6, 7, 9, 10 and 11 were produced with high efficiency in the soluble fraction. The molecular masses of each recombinant AtNUDT proteins agreed with their predicted molecular masses. Therefore, these recombinant proteins were purified using a HiTrap chelating HP column to apparent homogeneity, as judged with SDS-PAGE (Fig. 2). The production of recombinant proteins of AtNUDT8 was detected only in the insoluble fraction because of the formation of inclusion bodies.

Substrate specificities of recombinant AtNUDT proteins---To analyze the properties of cytosolic AtNUDTs as Nudiv hydrolases, we measured the hydrolytic activities of these enzymes in the presence of 5 mM Mg\textsuperscript{2+} for deoxyribonucleoside triphosphates and various types of nucleoside diphosphate derivatives with HPLC. The results are summarized in Table 1. The recombinant proteins of AtNUDT2, 6, 7 and 10 hydrolyzed both ADP-ribose and NADH to AMP. These proteins showed a high affinity for ADP-ribose compared with the other ADP-ribose pyrophosphatases,
hNUDT5 (25), Methanococcus jannaschii ADP-ribose pyrophosphatase, MJ1149 (23) and cyanobacterium Synechococcus sp. PCC 7002 ADP-ribose pyrophosphatase, NuhA (31) (Table 2). The AtNUDT2, 6 and 7 proteins also showed a high affinity for NADH.

AtNUDT1 was previously reported to have activity toward NADH in the presence of 5 mM Mn²⁺ (20). However, this activity was not detected in the presence of 5 mM Mg²⁺ (Table 1). In addition, AtNUDT1 showed hydrolysis activity with 5 mM Mg²⁺ toward 8-oxo-dGTP to 8-oxo-dGMP, with a high affinity (Table 3). Furthermore, the Ki value for 8-oxo-dGTP was lower than that of human MTH1 (34) and S. cerevisiae YLR151c (35), and we therefore designated it AtNUDT1/MutT1. Interestingly, the AtNUDT1 protein also showed a high affinity for dGTP, dATP and dTTP compared with other MutT-type enzymes (Table 3). The AtNUDT11 protein showed activity toward CoA-like mouse NudT7 (Nudt7-M), human NudT7 (Nudt7-H), and D. radiodurans DR-CoAase (2, 36). No activity to any of the substrates tested was detected in the AtNUDT4, 5 and 9 proteins.

Most of the characterized Nudix hydrolases require the presence of various divalent ions to become fully active. Therefore, we analyzed the requirement for divalent ions of AtNUDT1. Mg²⁺ was the most effective divalent ion. The presence of Mn²⁺ resulted in 79.5%–90.2% of the activity compared with the presence of Mg²⁺ (data not shown). Recently, Klaus et al. (2005) reported that the typical cytosolic levels of Mg²⁺ and Mn²⁺ were ~1 mM and ~1 μM, respectively, in plants (21). The AtNUDT1/MutT1 activities toward both 8-oxo-dGTP and other dNTPs in the presence of 1 mM Mg²⁺ were shown to be approx. 80% of those in 5 mM Mg²⁺ (data not shown). The activities in the presence of 1 μM Mn²⁺ were approx. 10% of those in 5 mM Mg²⁺. Similar results were observed in all of the other AtNUDT proteins. These results indicated that the divalent ion essential for AtNUDT1/MutT1 activity is Mg²⁺.

Complementation of the E. coli mutT mutation by AtNUDT/MutT1---Next, we examined the effects of the expression of AtNUDT1/MutT1 on the ratio of spontaneous mutation frequency toward streptomycin-resistance in the E. coli mutT strain, CC101T, which was devoid of its own 8-oxo-dGTPase (MutT) activity (9). As shown in Table 4, the mutation frequency of CC101T carrying pTrc100/AtNUDT1/MutT1 was completely suppressed to the same extent as that of the wild-type E. coli strain, CC101. However, the mutation frequency of CC101T cells carrying pTrc100/AtNUDT2 and 7 were almost the same as those of the cells with an empty pTrc100. These results clearly indicated that the AtNUDT1/MutT1 protein has the ability to prevent mutation via the sanitization of 8-oxo-dGTP, and thus acts as a functional homologue of E. coli MutT in A. thaliana.

Tissue-specific expression of AtNUDT transcripts---To confirm the tissue-specific expression of cytosolic AtNUDTs, we analyzed the transcript levels of cytosolic AtNUDTs in various tissues with the semi-quantitative RT-PCR. As shown in Fig. 3, the transcripts of all cytosolic AtNUDTs were detected in leaves, stems and roots. The transcript levels of AtNUDT6 in the roots were lower than those in leaves and stems. On the other hand, the transcript levels of AtNUDT3, 8 and 10 in leaves were lower than those in roots and stems. These results suggest that the expression of each AtNUDT is individually regulated in different tissues.

Discussion

The Nudix hydrolases are widely distributed in over 120 species ranging from viruses to humans (2). In A. thaliana, there were 3 types of Nudix hydrolases that were potentially localized in the cytosol, chloroplasts and mitochondria (Fig. 1). In eukaryotic cells, pools of their substrates, especially dNTPs, are present mainly in the cytosol (29) and human MTH1 is mostly
localized in the cytosolic fraction (30). Therefore, we analyzed here the molecular and enzymatic properties of cytosolic Nudix hydrolases.

AtNUDT1/MutT1, whose molecular weight (16.4 kDa) was very similar to that of E. coli MutT (14.9 kDa) and hMTH1 (17.9 kDa) had activities toward 8-oxo-dGTP with a higher affinity than those for other substrates (Tables 1 and 3). These results suggest that AtNUDT1/MutT1 is involved in the prevention of spontaneous mutation. In a mutT-deficient E. coli strain, the rate of spontaneous occurrence of A:T to C:G transversions increases to 1,000-fold that of the wild type level and also further increases transcriptional errors (6). Furthermore, hMTH1, homologues of E. coli MutT in human and rodent cells, can complement the function of MutT in E. coli (8-11). In addition, it has been reported that hNUDT5, which has activities toward not only 8-oxo-dGDP, but also ADP-ribose, suppresses the mutations of the E. coli mutT strain (38). Interestingly, AtNUDT1/MutT1 suppressed the mutation frequency of the E. coli mutT strain to the same extent as that in the wild-type E. coli strain (Table 4). Klaus et al. have recently suggested that AtNUDT1 is likely to be a bifunctional enzyme that may act as a MutT-type Nudix hydrolase and a dihydronopterin triphosphatase in the folate synthesis pathway (21). Our result clearly suggests that AtNUDT1/MutT1 functions as an 8-oxo-dGTPase in A. thaliana hydrolyzing 8-oxo-dGTP that accumulates in the nucleotide pool due to oxidative conditions. AtNUDT1/MutT1 was constitutively expressed in various tissues, suggesting that sanitization of the oxidized nucleotide pool in the cytosol may be necessary not only in photosynthetic tissues but also in non-photosynthetic ones.

However, the kinetic parameter of the enzymatic ability (kcat/Km) of AtNUDT1/MutT1 is approx. 20-fold lower than that of hMTH1; although, that is approx. 7-fold higher than that of YLR151c (Table 3). Interestingly, A. thaliana contained two different 8-oxo-G glycosylases (AtMMH, AtOgg1) encoded by the homologous genes of E. coli MutM, and yeast and human Ogg1, for the repair of 8-oxo-G from damaged DNA (39, 40). Therefore, the low ability of AtNUDT1 may be substantially complemented by the functions of AtMMH and AtOgg1. Furthermore, it is likely that some Nudix hydrolases localized in the chloroplasts and mitochondria may also function as a MutT-type enzyme to sanitize the nucleotide pool in each organelle.

On the other hand, AtNUDT2, 6, 7 and 10 had ADP-ribose pyrophosphatase activities with high affinity (Tables 1 and 2). Furthermore, the kinetic parameters of the enzymatic ability (kcat/Km) of these proteins were almost equal to those of hNUDT5 and MJ1149. Free ADP-ribose is produced during the reverse processes of degrading protein bound mono- or poly-(ADP-ribose) or cyclic ADP-ribose and is also produced by the turnover of β-NAD+ (41). ADP-riboosylation is a regulatory modification of protein in which an ADP-ribose moiety in β-NAD+ is transferred to a specific amino acid residue of the acceptor protein by poly (ADP-ribose) polymerase, in response to DNA strand break and resealing (43). The free ADP-ribose is a highly reactive molecule that causes non-enzymatic mono-ADP-riboosylation of proteins (42). Furthermore, mono-ADP-riboosylation of the protein by bacterial toxins leads to an immediate cytotoxic effect (44). Despite lacking knowledge about the possible specific roles of free ADP-ribose in cellular processes, it seems reasonable to conclude that the level of ADP-ribose in the cell should be carefully maintained to minimize the potential of the detrimental effect of free ADP-ribose (1, 23). Therefore, it is likely that the AtNUDTs (AtNUDT2, 6, 7 and 10) that have ADP-ribose pyrophosphatase activity can cooperatively play roles in removing the free ADP-ribose. In addition, AtNUDT2, 6, 7 and 10 also had NADH pyrophosphatase activity (Tables 1 and 2). It has been reported that NADH pyrophosphatase is involved in the regulation of the cellular NADH/NAD+ ratio, an important factor in maintaining the balance between the anabolic
and catabolic pathways in the cell (45), although the contribution of these AtNUDT proteins to the regulation is still unknown. AtNUDT6 was expressed at a low level in the roots, whereas AtNUDT2 and 7 were constitutively expressed in various tissues, suggesting that the expression of these enzymes with overlapping substrate spectra were individually regulated under different mechanisms (Fig. 3).

Interestingly, only AtNUDT11 had activity toward CoA, although the physiological function of the enzyme is not yet understood. Recently, it was reported that CoA and its derivatives are the substrates for Nudix hydrolase in D. radiodurans (2), mice and humans (36). All of them have the conserved motif, (LLTXR(SA)X3RX3GX3FPGG), which was found N-terminally adjacent to the Nudix motif. This motif was also conserved in AtMutT11 (Fig. 1).

There was no correlation between the conserved amino acid sequences and the enzymatic properties of the AtNUDT proteins, although the enzymes contained the Nudix motif, which was common among Nudix hydrolases in living organisms (Fig. 1 and Table 1). In addition, AtNUDT 2, 6, 7 and 10 showed an activity as the ADP-ribose pyrophosphatase, whereas these proteins had no proline residues at the 15 or 16th amino acid from the C-terminus glycine residue of the Nudix motif, which is characteristic of the subfamily of ADP-ribose pyrophosphatases (46). The results obtained here suggest that the plant Nudix family has evolved in a specific manner, different from that of yeast and human.
References
1. Bessman, M.J., Frick, D.N., & O’Handley, S.F. (1996) J. Biol. Chem. 271, 25059-25062
2. Xu, W., Shen, J., Dunn, C.A., Desai, S., & Bessman, M.J. (2001) Mol Microbiol. 39, 286-90
3. Dunn, C.A., O’Handley, S.F., Frick, D.N., & Bessman, M.J. (1999) J. Biol. Chem. 274, 32318-32324
4. Tajiri, T., Maki, H., & Sekiguchi, M. (1995) Mutat. Res. 336, 257-267
5. Maki, H., & Sekiguchi, M. (1992) Nature 355, 273-275
6. Taddei, F., Hayakawa, H., Bouton, M., Cirinesi, A., Matie, I., Sekiguchi, M., & Radman, M. (1997) Science 278, 128-130
7. Ames, B., Shigenaga, M., & Hagen, T. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7915-7922
8. Sakumi, K., Furuichi, M., Tsuzuki, T., Kakuma, T., Kawabata, S., Maki, H., & Sekiguchi, M. (1993) J. Biol. Chem. 268, 23524-23530
9. Furuichi, M., Yoshida, M. C., Oda, H., Tajiri, T., Nakabeppu, Y., Tsuzuki, T., & Sekiguchi, M. (1994) Genomics 24, 485-490
10. Cai, J. P., Kakuma, T., Tsuzuki, T., & Sekiguchi, M. (1995) Carcinogenesis 16, 2343-2350
11. Kakuma, T., Nishida, J., Tsuzuki, T., & Sekiguchi, M. (1995) J. Biol. Chem. 270, 25942-25948
12. Hays, J.B. (2002) DNA Repair 1, 579-600
13. Asada, K. (1997) (Scandalios, J.G., ed). New York: Cold Spring Harbor Laboratory Press, pp. 715-735
14. Foyer, C.H., Descourvières, P. & Kunert, K.J. (1994a) Plant Cell Environ. 17, 507-523
15. Alscher, R. G., Donahue, J.L., & Cramer, C.L. (1997) Physiol. Plant 100, 224-233
16. Asada K (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 601-639
17. Bowler, C. Montagu, M.V. & Inzé, D. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 83-116
18. Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, & Yoshimura K (2002) J. Exp. Bot. 53, 1305-1319
19. Altschul, S.F., Gish, W., Meyers, E.W., & Lipman, D.J. (1990) J. Mol. Biol. 203, 403-410
20. Dobrzanska, M., Szurmak, B., Wyslouch-Cieszynska, A., & Kraszewska, E. (2002) J. Biol. Chem. 277, 50482-50486
21. Klaus, S.M., Wegkamp, A., Sybesma, W., Hugenholtz, J., Gregory, J.F. 3rd., & Hanson, A.D. (2005) J. Biol. Chem. 280, 5274-5280
22. Kwak, Y.-G., Park, S.-K., Kim, U.-H., Han, M.-K., Eun, J.-S., Cho, K.-P., & Chae, S.-W. (1996) An.J.Physiol. 271, C464-C468
23. Sheikh, S., O’Handley, S.F., Dunn, C.A., & Bessman, M.J. (1998) J. Biol. Chem. 273, 20924-20928
24. O’Handley, S.F., Frick, D.N., Dunn, C.A., & Bessman, M.J. (1998) J. Biol. Chem. 273, 3192-3197
25. Yang, H., Slupska, M.M., Wei, Y.F., Tai, J.H., Luther, W.M., Xia, Y.R., Shih, D.M., Chiang, J.H., Baikalov, C., Fitz-Gibbon, S., Phan, I. T., Conrad, A., & Miller, J.H. (2000) J. Biol. Chem. 275, 8844-8853
26. Yoshimura, K., Yabuta, Y., Tamoi, M., Ishikawa, T., & Shigeoka, S. (1999) Biochem. J. 338, 41-48
27. Bradford, M. (1976) Anal. Biochem. 72, 248-254
28. Tassotto, M.L. & Mathews, C.K. (2002) J. Biol. Chem. 277, 15807-15812
29. Bestwick, R.K., Moffett, G.M., & Mathews, C.K. (1982) J. Biol. Chem. 257, 9300-9304
30. Kang, D., Nishida, J., Iyama, A., Nakabeppu, Y., Furuichi, M., Fujiwara, T., Sekiguchi, M., & Takeshige, K. (1995) J. Biol. Chem. 270, 14659-14665
31. Okuda, K., Nishiyama, Y., Morita, E.H., & Hayashi, H. (2004) *Biochim. Biophys. Acta* **1699**, 245-252
32. Abdelraheim, S.R., Cartwright, J.L., Gasmi, L., & McLennan, A.G. (2001) *Arch Biochem Biophys.* **388**, 18-24
33. Abdelraheim, S.R., Spiller, D.G., McLennan, A.G. (2003) *Biochem J.* **374**, 329-35
34. Fujikawa, K., Kamiya, H., Yakushiji, H., Fujii, Y., Nakabeppu, Y., & Kasai, H. (1999) *J. Biol. Chem.* **274**, 18201-18205
35. Nunoshiba, T., Ishida, R., Sasaki, M., Iwai, S., Nakabeppu, Y., & Yamamoto, K. (2004) *Nucleic Acids Res.* **32**, 5339-5348
36. Gasmi, L. & McLennanm, A.G. (2001) *Biochem. J.* **357**, 33-38
37. Furuichi, M., Yoshida, M. C., Oda, H., Tajiri, T., Nakabeppu, Y., Tsuzuki, T., & Sekiguchi, M. (1994) *Genomics* **24**, 485-490
38. Ishibashi T, Hayakawa H, & Sekiguchi M. (2003) *EMBO reports.* **4**, 479-483
39. Ohtsubo, T., Matsuda, O., Iba, K., Terashima, I., Sekiguchi, M., & Nakabeppu, Y. (1998) *Mol Gen Genet.* **259**, 577-90
40. Garcia-Ortiz, M.V., Ariza, R.R., & Roldan-Arjona, T. (2001) *Plant Mol Biol.* **47**, 795-804
41. Olivera, B.M., Hughes, K.T., Cordray, P., & Roth, J.R. (1989) in *ADP-ribose transfer Reactions.* (Jacobson, M.K., and Jacobson, E.L., eds) pp. 353-360, Springer-Verlag, New York
42. Jacobson, E.L., Cervantes-Laurean, D., & Jacobson, M.K. (1994) *Mol. Cell. Biochem.* **138**, 207-212
43. Amé, J.C., Rolli, V., Schreiber, V., Niedergang, C., Apiou, F., Decker, P., Muller, S., Höger, T., Ménissier-de Murcia, J., & de Murcia G. (1999) *J. Biol. Chem.* **274**, 17860-17868
44. MacDonald, L.J., & Moss, J. (1994) *Mol Cell. Biochem.* **138**, 221-226
45. Frick, D.N., & Bessman, M.J. (1995) *J. Biol. Chem.* **270**, 1529-1534
46. Lin, S., Gasmi, L., Xie, Y., Ying, K., Gu, S., Wang, Z., Jin, H., Chao, Y., Wu, C., Zhou, Z., Tang, R., Mao, Y., & McLennan, A.G. (2002) *Biochem. Biophys. Acta* **1594**, 127-135
Footnotes
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Figure legends
Fig. 1. Partial sequence alignment of AtNUDT proteins with ADP-ribose and 8-oxo-dGTP pyrophosphatases of the Nudix family. Polypeptides identified in a Blast search against the Nudix signature sequences are shown. Amino acids that are fully conserved or substitutive are shown by grey box. The Nudix motif is shown below the sequence. The sequences used here are as follows AtNUDT1, At1g68760; AtNUDT2, At5g47650; AtNUDT3, At1g79690; AtNUDT4, At1g18300; AtNUDT5, At2g04430; AtNUDT6, At2g04450; AtNUDT7, At4g12720; AtNUDT8, At5g47240; AtNUDT9, At3g46200; AtNUDT10, At4g25434; AtNUDT11, At5g45940; AtNUDT12, At1g12880; AtNUDT13, At3g26690; AtNUDT14, At4g11980; AtNUDT15, At1g28960; AtNUDT16, At3g12600; AtNUDT17, At2g01670; AtNUDT18, At1g14860; AtNUDT19, At5g20070; AtNUDT20, At5g19460; AtNUDT21, At1g73540; AtNUDT22, At2g33980; AtNUDT23, At2g42070; AtNUDT24, At5g19470; *E. coli* MutT, P08337; *Homo sapiens* MTH1, P36639; *Homo sapiens* NUDT5, AF218818; *Mus musculus* Nudt7, AF338424; *Synechococcus* sp. PCC7002 nuaA, AB105878; *Methanococcus jannaschii* MJ1149, D64443; *S. cerevisiae* YLR151c, NP_013252.

Fig. 2. Purification of recombinant AtNUDT proteins. Recombinant AtNUDT proteins were overexpressed in *E. coli*, purified with Ni$^{2+}$ affinity chromatography, and verified using SDS-PAGE with Coomassie blue staining. The experimental conditions are described in “EXPERIMENTAL PROCEDURES”. Left lanes and right lanes of all AtNUDTs contain 15 µg crude extract and 2 µg of purified recombinant proteins, respectively. M: molecular mass standards (Amersham Bioscience) as indicated on the left.

Fig. 3 Expression of the cytosolic *AtNUDT* genes in different plant tissues. Semi-quantitative RT-PCR was performed using specific primers for *AtNUDT* genes and *Actin8* on total RNA from roots, stems and leaves. PCR amplification was performed with 20–26 cycles of 95°C for 60s, 55°C for 60s, and 72°C for 60s, followed by 72°C for 10 min. Aliquots of the products were analysed on 1% agarose gel.
### Table 1  Substrate specificities of AtNUDT proteins

| Substrate       | AtNUDT1 | AtNUDT2 | AtNUDT4 | AtNUDT5 | AtNUDT6 | AtNUDT7 | AtNUDT9 | AtNUDT10 | AtNUDT11 |
|------------------|---------|---------|---------|---------|---------|---------|---------|----------|----------|
| 8-oxo-dGTP       | 0.94 ± 0.04 | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.     | n.d.     |
| dGTP             | 1.48 ± 0.07 | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.     | n.d.     |
| dATP             | 0.65 ± 0.04 | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.     | n.d.     |
| dTTP             | 0.90 ± 0.03 | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.     | n.d.     |
| dCTP             | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.     | n.d.     |
| NADH             | n.d.    | 0.10 ± 0.01 | n.d.    | n.d.    | 0.28 ± 0.03 | 0.07 ± 0.01 | n.d.    | < 0.01   | n.d.     |
| ADP-ribose      | n.d.    | 0.19 ± 0.02 | n.d.    | n.d.    | 0.19 ± 0.01 | 0.11 ± 0.01 | n.d.    | 0.08 ± 0.02 | n.d.     |
| ADP-glucose     | n.d.    | < 0.01   | n.d.    | n.d.    | n.d.    | 0.02 ± 0.01 | n.d.    | < 0.01   | n.d.     |
| Ap3A            | n.d.    | 0.02 ± 0.01 | n.d.    | n.d.    | < 0.01   | < 0.01   | n.d.    | < 0.01   | n.d.     |
| Ap4A            | n.d.    | 0.02 ± 0.01 | n.d.    | n.d.    | < 0.01   | 0.02 ± 0.01 | n.d.    | < 0.01   | n.d.     |
| Ap5A            | n.d.    | < 0.01   | n.d.    | n.d.    | n.d.    | < 0.01   | n.d.    | < 0.01   | n.d.     |
| UDP-glucose     | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.     | n.d.     |
| UDP-galactose   | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.     | n.d.     |
| CoA             | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.    | n.d.     | 0.42 ± 0.01 |
| FAD             | n.d.    | 0.06 ± 0.01 | n.d.    | n.d.    | < 0.01   | 0.02 ± 0.01 | n.d.    | < 0.01   | n.d.     |

All substrates were at a concentration of 25 µM and 0.5–1 µg of the recombinant proteins were used. The activities of the recombinant AtNUDT proteins were measured at 37 °C with 5 mM Mg²⁺, as described in “EXPERIMENTAL PROCEDURES”. Data are the means of 3 independent determinations ± S.D. Ap₃A, adenosine (5’)-triphospho (5’) adenosine. Other dinucleoside polyphosphates are abbreviated in an analogous manner. All specific activities were µmol/min/mg. n.d.; not detected.
Table 2  *The kinetic parameters of ADP-ribose and NADH pyrophosphatases in A. thaliana, human, M. jannaschii, Synechococcus PCC7002, and yeast*

| Protein | \(K_m\) \(\mu M\) | \(V_{max}\) \(\mu mol/min/mg\) | \(k_{cat}\) \(s^{-1}\) | \(k_{cat}/K_m\) \(s^{-1} M^{-1}\) |
|---------|----------------|-----------------|----------------|-----------------|
| ADP-ribose | | | | |
| AtNUDT2 | 16.9±2.3 | 0.20±0.01 | 0.12 | 7.0 \(\times\) 10³ |
| AtNUDT6 | 23.0±0.9 | 0.18±0.01 | 0.11 | 4.6 \(\times\) 10³ |
| AtNUDT7 | 23.2±6.3 | 0.24±0.02 | 0.12 | 5.3 \(\times\) 10³ |
| AtNUDT10 | 27.4±2.1 | 0.10±0.01 | 0.06 | 6.3 \(\times\) 10³ |
| MJ1149 | 31.6±5.1 | 9.50±2.11 | - | - |
| NuhA | 94 | 23.6 | 1.8 | 1.9 \(\times\) 10⁴ |

| NADH | | | | |
| AtNUDT2 | 22.3±1.1 | 0.16±0.01 | 0.01 | 4.3 \(\times\) 10³ |
| AtNUDT6 | 13.7±0.4 | 0.32±0.01 | 0.18 | 1.4 \(\times\) 10⁴ |
| AtNUDT7 | 37.2±5.3 | 0.09±0.01 | 0.06 | 1.5 \(\times\) 10³ |
| NPY1 | 170 | 2.0 | 1.5 | 8.5 \(\times\) 10³ |
| hNUDT12 | 11 | 12.0 | 11.0 | 1.0 \(\times\) 10⁶ |

The standard assay was used with concentrations of 5-300 \(\mu M\) for ADP-ribose and NADH at 37 °C with 5 mM Mg\(^{2+}\) as described in “EXPERIMENTAL PROCEDURES”. Data are the means of 3 independent determinations ± S.D.

\(a\) Yang *et al.* (25); \(b\) Sheikh *et al.* (23); \(c\) Okuda *et al.* (31); \(d, e\) Abdelraheem *et al.* (32, 33).
Table 3 Comparison of the kinetic parameters of AtNUDT1/MutT1 in A. thaliana with those of MutT-like proteins in E. coli, human, and yeast

| Protein            | Km   | V<sub>max</sub> | k<sub>cat</sub> | k<sub>cat</sub>/Km |
|--------------------|------|----------------|----------------|-------------------|
|                    | µM   | µmol/min/mg    | s<sup>-1</sup>  | s<sup>-1</sup>M<sup>-1</sup> |
| AtNUDT1            |      |                |                |                   |
| 8-oxo-dGTP         | 6.8±0.9 | 0.8±0.1   | 0.25           | 3.7 x 10<sup>4</sup> |
| dGTP               | 58.3±2.5 | 2.7±0.2   | 0.83           | 1.4 x 10<sup>4</sup> |
| dATP               | 16.1±1.3 | 5.8±0.1   | 0.17           | 1.1 x 10<sup>4</sup> |
| dTTP               | 15.6±3.0 | 0.8±0.1   | 0.25           | 1.5 x 10<sup>4</sup> |
| E. coli MutT<sup>a</sup> |      |            |                |                   |
| 8-oxo-dGTP         | 0.48 | 4.2         | -              | -                 |
| dGTP               | 1100 | 4.8         | -              | -                 |
| dATP               | 1800 | 1.3         | -              | -                 |
| dTTP               | 1700 | 0.28        | -              | -                 |
| hMTH1<sup>b</sup>  |      |            |                |                   |
| 8-oxo-dGTP         | 15.2 | -           | 12.3           | 8.0 x 10<sup>5</sup> |
| dGTP               | 870  | -           | -              | -                 |
| YLR151c<sup>c</sup> |      |            |                |                   |
| 8-oxo-dGTP         | 23.8 | -           | 0.13           | 5.6 x 10<sup>5</sup> |

The standard assay was used with concentrations of 5-300 µM for all substrates at 37 °C with 5 mM Mg<sup>2+</sup> as described in “EXPERIMENTAL PROCEDURES”. Data are the means of 3 independent determinations ± S.D. <sup>a</sup> Maki et al. (5), <sup>b</sup> Fujikawa et al. (34), and <sup>c</sup> Nunoshiba et al. (35).
### Table 4  Suppression of E. coli mutT mutator activity by the expression of AtNUDT1/MutT1

| E. coli strain | Mutation frequency | Relative ratio |
|----------------|--------------------|----------------|
| CC101 (wild-type) | $2.24 \pm 0.55 \times 10^{-10}$ | 1.0 |
| CC101T (mutT) with pTrc100 (vector) | $5.46 \pm 0.99 \times 10^{-7}$ | 2438 |
| CC101T (mutT) with pTrc100/AtNUDT1/MutT1 | $1.31 \pm 0.90 \times 10^{-9}$ | 5.8 |
| CC101T (mutT) with pTrc100/AtNUDT2 | $5.90 \pm 1.31 \times 10^{-7}$ | 2634 |
| CC101T (mutT) with pTrc100/AtNUDT7 | $4.38 \pm 0.39 \times 10^{-7}$ | 1955 |

The mutation frequency was calculated according to the number of colonies on the streptomycin plates, as described in “EXPERIMENTAL PROCEDURES”. Wild-type cells and mutT cells are the *E. coli* strains CC101 and CC101T, respectively, and carry either pTrc100 (vector), pTrc100::AtNUDT1, pTrc100::AtNUDT2, or pTrc100::AtNUDT7. Data are the means of 3 independent determinations ± S.D.
Fig. 1

Nudix motif
Fig. 2
Fig. 3
