A Comparative Analysis of the Molecular Characteristics of the Arabidopsis CoA Pyrophosphohydrolases AtNUDX11, 15, and 15a

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Received August 25, 2011; Accepted October 4, 2011; Online Publication, January 7, 2012
[doi:10.1271/bbb.110636]

Coenzyme A (CoA) is an essential, ubiquitous cofactor in all biological systems, where it acts as the major acyl group carrier in various central metabolic reactions. Although much is known about CoA biosynthesis, it is unclear how the CoA pool is regulated in the various cellular compartments. It has been found that the nucleoside diphosphates linked to some moiety X (Nudix) hydrolases, AtNUDX11 and 15, have pyrophosphohydrolase activity toward CoA and its derivatives. In this study we identified two alternatively spliced variants, AtNUDX15 and 15a, produced from the AtNUDX15 gene, and carried out comparative studies of the gene regulation, the kinetic parameters, and the intracellular localization of AtNUDX11, 15, and 15a. The present findings indicate that AtNUDX11 and AtNUDX15(a) function in the hydrolysis of malonyl-CoA in cytosol and succinyl-CoA in the mitochondria, respectively, suggesting their impact not only on CoA biosynthesis but also on various CoA-related pathways such as the TCA cycle.

Key words: Arabidopsis; coenzyme A; nucleoside diphosphates linked to some moiety X (Nudix) hydrolase

CoA is required for the production and degradation of both primary and secondary metabolites in bacteria, plants, and animals, where it is involved in more than 100 different reactions.11 It is synthesized from panthothenate via five enzymatic reactions, catalyzed by panthothenate kinase (PANK), 4'-phospho-N-pantothenoyl-cysteine (PPC) synthase (PPCS), PPC decarboxylase (PPCD), 4'-phosphopantetheinyl adenyltransferase (PPAT), and dephospho-CoA kinase (DPCK).1–7 The activity of PANK and PPAT is known to be controlled by the levels of CoA and CoA thioesters, the end-products of CoA biosynthesis.3–8) The 4'-phosphopantetheinyl (P-pant) moiety of CoA can be transferred to a serine residue of acyl carrier proteins (ACPs) by holo-ACP synthase (ACPS).14–16) The resulting holo-ACP is then active as a central coenzyme of fatty acid biosynthesis. CoA is degraded by CoA pyrophosphohydrolase, which belongs to the family of nucleoside diphosphates linked to some moiety X (Nudix) hydrolase, by which 4'-phosphopantetheine and 3',5'-ADP are produced.17–24)

Nudix hydrolases with pyrophosphatase activity toward nucleoside diphosphate derivatives are characterized by a conserved motif (Nudix box, PROSITE PS00893) consisting of 23 amino acids, GX2EX2-REXEEUXGU, where U is an aliphatic, hydrophobic residue, although several interesting examples exist with altered consensus sequences.25–27) Humans and Arabidopsis plants possess 24 (NUDT1-22) and 28 (AtNUDX1-27 and AtDP2) types respectively of Nudix hydrolases, which differ in substrate specificity and subcellular localization.22,23,27,28) Accordingly, the enzymes have activity hydrolyzing nucleoside di- and tri-phosphates and their oxidized forms, dinucleoside polyphosphates, nucleotide sugars, NADH, and also dinucleoside tri-phosphates and their oxidized forms, dinucleoside polyphosphates, nucleotide sugars, NADH, and also Nudix diphosphates (NDPs), the mRNA cap, 5'-triphosphorylated RNA, and guanosine 3',5'-bisphosphopentofuranosyl (ppGpp), and non-nucleoside substrates such as diphosphoinositol polyphosphates (DIPs), 5-phosphoribosyl 1-diphosphate (PRPP), thiamine pyrophosphate (TPP), and dihydroxyacetone phosphatase (DHNP) are also recognized as substrates for Nudix hydrolases.29,30 All these substrates are either potentially toxic compounds, cell-signaling molecules, or metabolic intermediates, the concentrations of which must be closely regulated during the cell cycle and the growth period, and/or in response to environmental conditions. Hence it has been postulated that the role of Nudix hydrolases is to suppress or maintain the levels of such molecules.25)

Nudix hydrolases with pyrophosphohydrolase activity toward CoA and its derivatives, such as acetyl-CoA and succinyl-CoA, have been identified in various organ-

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Abbreviations: ACP, acyl carrier protein; ACPS, ACP synthase; CoA, coenzyme A; DHNTP, dihydroxyacetone triphosphate; DIP, diphosphoinositol polyphosphate; DPCK, dephospho-CoA kinase; EST, expressed sequence tag; GFP, green fluorescent protein; mTP, mitochondrial targeting peptide; NADPH-GAPDH, NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase; NDP, nucleoside diphosphate; Nudix, nucleoside diphosphates linked to some moiety X; PANK, panthothenate kinase; P-pant, 4'-phosphopantetheinyl; PPAT, 4'-phosphopantetheinyl adenyltransferase; PPC, 4'-phospho-N-pantothenoyl-cysteine; PPCD, PPC decarboxylase; PPCS, PPC synthase; ppGpp, guanosine 3',5'-bispyrophosphate; PTS1, type-1 peroxisomal targeting signal; SDH, succinate dehydrogenase; SPT, serine:pyruvate aminotransferase

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isms, including yeasts (Saccharomyces cerevisiae), nematodes (Caenorhabditis elegans), mice, Arabidopsis thaliana, and plants (Arabidopsis thaliana). Suggesting that these enzymes are well conserved in various organisms across the evolutionary process. This strongly suggests the importance of CoA pyrophosphohydrolase in the metabolism of CoA and its derivatives. In fact, it has been reported that the expression of mouse Nudt7α, a peroxisomal Nudix hydrolase having pyrophosphohydrolase activity not only toward CoA but also toward acyl-CoAs, is negatively correlated with the levels of CoA as well as the expression of the enzymes involved in peroxisomal lipid metabolism. These findings suggest that Nudt7α is tightly associated with the homeostasis of CoA/acyl-CoA in the peroxisomes. The Arabidopsis Nudix hydrolases AtNUDX11 and AtNUDX15, which are predicted to occur in the cytosol and the mitochondria respectively, had pyrophosphohydrolase activity toward CoA and its derivatives, including not only malonyl-CoA and succinyl-CoA, metabolites of fatty acid biosynthesis in chloroplasts and the TCA cycle in mitochondria, but also oxidized CoA, a potentially toxic or nonfunctional form of CoA. Notably, AtNUDX11-overexpressing Arabidopsis plants showed enhanced growth and an extended life cycle as compared to wild-type plants. Hence it has been suggested that the action of AtNUDX11 is implicated in embryogenesis through the elimination of toxic or useless CoA derivatives. However, knowledge of the physiological functions of CoA pyrophosphohydrolase is still limited, since there is no direct evidence linking the actions of the enzyme with cellular processes.

In this study, we found that the gene encoding AtNUDX15 produced alternatively spliced variants (designated AtNUDX15 and AtNUDX15a). Hence, to gain further insight into the physiological roles of plant CoA pyrophosphohydrolases, the regulatory system for gene expression, the kinetic parameters of enzymatic activity, and the intracellular localization of AtNUDX11, 15, and 15a were investigated. The results obtained here suggest the importance of these enzymes in the regulation of the metabolism of CoA derivatives in the cytosol and mitochondria of plant cells.

**Materials and Methods**

**Materials and plant growth conditions.** Arabidopsis plants (ecotype Columbia) were grown on basic Murashige and Skoog (MS) medium in petri dishes containing 3% (w/v) sucrose for 2 weeks, and then grown in soil at 25°C under long-day conditions (16h light at 100μEm−2 s−1/8h dark).

The vectors for the Gateway cloning system, pDONR201, and pGWB2, 5, and 6, were obtained from Dr. T. Nakagawa (Shimane University). 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.

**Analysis of AtNUDX expression in various organs.** Total RNA was isolated from various organs (0.5 g of fresh weight), rosette leaves, stems, cauline leaves, inferences, siliques, and roots of 6-week-old Arabidopsis plants, as described previously. First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) with an oligo (dT) primer as described above. cDNAs encoding the various AtNUDXs and Actin2 were semi-quantitatively amplified by PCR. The specific primer sets used in the analysis were as follows (Supplemental Table 1; see Biosci. Biotechnol. Bioch. Web site): AtNUDX11, AtNUDX11-F and AtNUDX11-R; AtNUDX15a, AtNUDX15a-F and AtNUDX15a-R; Actin2, Actin2-F and Actin2-R. Equal loading of the various amplified gene sequences was determined with the control Actin2 PCR product.

**Expression and purification of the recombinant AtNUDX15a protein.** cDNA fragments encoding the mature AtNUDX15a protein, except for the predicted mitochondrial transit peptides at the N-terminus, were amplified by PCR from first-strand cDNA synthesized from the total RNA of Arabidopsis using specific primers AtNUDX15a-5cH-F and AtNUDX15a-5cH-R (Supplemental Table 1). The amplified DNA fragment was ligated into pPT Blue T-vector, digested with the desired restriction enzymes, and re-cloned into vector pCold II (Takara). A recombinant form of AtNUDX15a was produced using E. coli strain BL21 (DE3) pLysS cells and was purified from the extract using a HiTrap chelating HP column (GE Healthcare, Little Chalfont, UK) following the manufacturer’s instructions. The production and purification of the recombinant AtNUDX11 and 15 were carried out following previous reports. The protein content was determined by the Bradford method, using bovine serum albumin (BSA) as standard. The molecular masses of the recombinant AtNUDX11, 15, and 15a proteins agreed with the predicted values, calculated from the amino acid sequence of the mature protein plus the hexahistidine-tag (AtNUDX11; 27.9 kDa, AtNUDX15; 32.6 kDa, AtNUDX15a; 31.7 kDa).

**Enzyme assay and HPLC.** The hydrolytic activities of recombinant forms of AtNUDX11, 15, and 15a toward CoA derivatives were assayed by a method described previously. Sixty μL of the reaction mixture, containing 50 mM Tris–HCl (pH 8.0), 5 mM MgCl2, 5–3,000 μM substrate, and 0.2–1.0 μg of the purified recombinant protein, 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 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. It contained 73 mM KH2PO4, 5 mM tetraethylammonium dihydrogenophosphate, and 20% methanol. The substrates (CoA, succinyl-CoA, malonyl-CoA, lauroyl-CoA, myristoyl-CoA, and oxidized-CoA) and the reaction product (3',5'-ADP) were detected by their UV absorbance at 260 nm. Leaves (0.3 g) of the Arabidopsis plants were homogenized with 1 mL of 100 mM Tris–HCl (pH 8.0) containing 20% glycerol. After centrifugation (20,000 × g) for 20 min at 4°C, the supernatant was used for analysis of enzymatic activity. Crude extracts (approximately 10 μg of protein) were used in this assay.

**Subcellular localization of the green fluorescent protein (GFP) fusion protein.** The vectors for the generation of GFP-fused proteins were constructed using GATEWAY cloning technology (Invitrogen, Carlsbad, CA, USA). The cDNAs encoding the open reading frames of the AtNUDXs were cloned into the donor vector, pDONR201, and then re-cloned into the destination vectors pGWB6 or pGWB5, in which the AtNUDX proteins, fused with GFP at N- or C-terminus, were expressed under the control of the cauliflower mosaic virus 35S promoter. PCR and in vitro BP and LR recombination reactions were carried out following the manufacturer’s instructions (Invitrogen). The specific primer sets were as follows (Supplemental Table 1): AtNUDX11-cGFP, atb1-AtNUDX11-cGFP and atb2-AtNUDX11-cGFP; AtNUDX15-cGFP, atb1-AtNUDX15-cGFP and atb2-AtNUDX15-cGFP; AtNUDX15a-cGFP, atb1-AtNUDX15a-cGFP and atb2-AtNUDX15a-cGFP; AtNUDX15-nGFP, atb1-AtNUDX15-15a-nGFP and atb2-AtNUDX15-15a and atb2-AtNUDX15a-cGFP; AtNUDX15-nGFP, atb1-AtNUDX15-15a-nGFP and atb2-AtNUDX15-15a and atb2-AtNUDX15a-cGFP; AtNUDX15-nGFP, atb1-AtNUDX15-15a-nGFP and atb2-AtNUDX15-15a. To fuse the GFP cDNA in-frame with the cDNA encoding AtNUDX15 or 15a in the internal regions, pDONR/AtNUDX15 or 15a was digested with DruI, by which AtNUDX15 (882 bp) and 15a (858 bp) cDNAs are separated by which AtNUDX15 (882 bp) and 15a (858 bp) cDNAs are separated fragments into 2 fragments (291 and 591 bp, and 291 and 567 bp, respectively), and dephosphorylated by Alkaline phosphatase, SAP (Takara, Kyoto, Japan) (Supplemental Fig. 1). Amplified GFP cDNA using phosphorylated primers (GFP-F and GFP-R) (Supplemental Table 1) were ligated into linearized pDONR/AtNUDX15 or 15a. The nucleotide sequence was verified by DNA sequencing. The fragment obtained was re-
cloned into the destination vector, pGWB2. Agrobacterium tumefaciens (strain C58), which was transformed with constructs obtained by electroporation, was used in the transformation of Nicotiana tabacum BY-2 (Nicotiana tabacum L. cv bright yellow 2) cells by a modification of the procedure reported by Gu and Verma.22) The fluorescence of the GFP fusion proteins in protoplasts prepared from transgenic BY-2 cells was monitored using a Radiance 2100 confocal fluorescence microscope (Bio-Rad, Hercules, CA).23) The mitochondria were stained with a mitochondria-selective dye, MitoTracker Orange (Invitrogen). Photographs were processed using LaserSharp2000 software (Carl Zeiss, Oberkochen, Germany).

Protein analysis. A polyclonal mouse antibody raised against the AtNUDX15 protein was prepared using His-tagged recombinant AtNUDX15, as described previously.22) Immunoblot analysis was performed as reported previously.42) AtNUDX11, 15, 15a, and 22 proteins were detected using the AtNUDX15 antibody as primary antibody and anti-mouse IgG-horseradish peroxidase conjugate (Bio-Rad) as secondary antibody. Protein bands were detected using the enhanced chemiluminescence detection system (GE Healthcare). The protein concentration was determined by the method of Bradford.40) using BSA as standard.

Generation of transgenic plants. Total RNA was isolated from the leaves of 2-week-old Arabidopsis plants (0.5 g fresh weight), as described previously.43) First-strand cDNA was synthesized using ReverTra Ace reverse transcriptase (Toyobo) with an oligo (dT) primer. The vector for generating the AtNUDX15- and 15a-overexpressing plants was constructed using Gateway cloning technology (Invitrogen). The cDNAs encoding the open reading frame of AtNUDX15 and 15a were cloned into the donor vector, pDONR201, and then recloned into the destination vector, pGWB2. The specific primer sets were as follows (Supplemental Table 1): AtNUDX15, attB1-AtNUDX15-15a and attB2-AtNUDX15, attB1-AtNUDX15-15a and attB2-AtNUDX15a, PCR and in vitro BP and LR recombination reactions were performed following the manufacturer’s instructions (Invitrogen). Agrobacterium tumefaciens, which was transformed with the constructs obtained by electroporation, was used to infect Arabidopsis by the vacuum infiltration method. T1 seedlings were selected on basic MS medium in petri dishes containing 3% sucrose, 20 mg L−1 of hygromycin, and 20 mg L−1 of kanamycin over 2 weeks, and then transferred to soil. Homozygous T2 seeds were harvested and used in the experiments.

Isolation of organelles from Arabidopsis leaves. Six-week-old Arabidopsis plants were transferred to dark conditions for induction of senescence, and then grown for 4 d by the method described by Reumann et al.44) Organelles were isolated from Arabidopsis (20 g FW) using Percoll (15–38%) and sucrose (0–36%) density gradients (10 mL). After centrifugation, 1-mL fractions were isolated. The activities of catalase,45) succinate dehydrogenase (SDH),46) and NADPH-dependent glycerolaldehyde-3-phosphate dehydrogenase (NADPH-GAPDH)47) were measured as markers of peroxisomes, mitochondria, and chloroplasts respectively.

Results and Discussion

Alternative splicing of AtNUDX15

It has been reported that among Arabidopsis Nudix hydrolases, AtNUDX11 and 15 have CoA pyrophosphohydrolase activity.22,23) AtNUDX11 was predicted to be localized to the cytosol, since it had no transit peptide as judged by TargetP (www.cbs.dtu.dk/services/TargetP/). On the other hand, AtNUDX15 had the mitochondrial targeting peptide (mTP) at the N-terminus. The mitochondrial localization was verified using a GFP-fused protein.23) A search of the expressed sequence tag (EST) database identified a new alternatively spliced variant (designated AtNUDX15a) produced from the AtNUDX15 gene (Fig. 1A). Due to intron retention-type alternative splicing, the AtNUDX15a mRNA, which is shorter than the AtNUDX15 mRNA, was produced. Translation of the AtNUDX15 mRNA was predicted to stop at the UAA codon, resulting in the production of an AtNUDX15 protein composed of 294 amino acids (Fig. 1B). On the other hand, the AtNUDX15a mRNA translated a protein composed of 286 amino acids due to usage of the alternative stop (UGA) codon. Semi-quantitative RT-PCR indicated that AtNUDX15 and 15a mRNAs were both expressed in the leaves of 2-week-old Arabidopsis plants (Fig. 2). The nucleotide sequences of the various mRNAs were verified by DNA sequencing. The deduced AtNUDX15 and 15a proteins have the Nudix motif and the UPF0035 motif conserved in CoA pyrophosphohydrolases.19,20,23) Importantly, the predicted amino acid sequence of AtNUDX15a has the type-1 peroxisomal targeting signal (PTS1) at the C-terminus, in addition to the mTP at the N-terminus, like AtNUDX15. In other words, AtNUDX15a has mTP and PTS1 at the N- and the C-terminus respectively, suggesting a dual subcellular distribution in mitochondria and peroxisomes.

The expression of AtNUDX11, 15, and 15a in various plant organs

Semi-quantitative RT-PCR was used to examine the expression of the AtNUDX11, 15, and 15a mRNAs in rosette leaves, stems, cauline leaves, inflorescences, siliques, and roots. AtNUDX11 mRNA was detected in all the organs tested, but levels were highest in the inflorescences (Fig. 2). Consistent with this result, Kupke et al.39) reported that AtNUDX11 expression is ubiquitous, but strong in flowers, vascular elements, and developing embryos. More detailed information on the expression of AtNUDX11 in the floral organs was obtained from the microarray data bank Genevestigator.48) with higher levels of AtNUDX11 observed in petals, stigmas, and abscission zones (Supplemental Fig. 2). Like AtNUDX11, AtPANK1 and 2, enzymes for CoA biosynthesis, were highly expressed in these organs. Hence, it is likely that the function of AtNUDX11 is associated with CoA biosynthesis in floral development.

AtNUDX15 mRNA was expressed in the stems, roots, and rosette and cauline leaves, but not in the inflorescences or siliques. The levels of AtNUDX15 mRNA were similar to those of AtNUDX15a mRNA in the stems, roots, and rosette and cauline leaves, but AtNUDX15a mRNA was detected in the inflorescences (Fig. 2). These results indicate that the alternative splicing of AtNUDX15 is regulated in an organ-specific manner. Although microarray analysis cannot independently detect alternatively spliced variants, the data from Genevestigator indicates that the AtNUDX15 or 15a mRNA is highly expressed in the pollen, suggesting that the proteins are involved in the male reproductive organs.

Enzymatic characterization of AtNUDX11, 15, and 15a

To characterize the recombinant forms of AtNUDX15a, the His-tagged AtNUDX15a gene product was expressed in E. coli transformed with the pColdII/AtNUDX15a plasmid. Affinity chromatography on a HiTrap chelating column yielded a highly purified recombinant protein, as found on the SDS/PAGE analysis (Fig. 3). The hydrolytic activities of this
enzyme toward various nucleoside diphosphate derivatives were determined by HPLC. AtNUDX15a hydrolyzed CoA and its derivatives, including malonyl-CoA, succinyl-CoA, oxidized-CoA, lauroyl-CoA (12:0-CoA), and myristoyl-CoA (14:0-CoA) (Table 1). A product, 3',5'-ADP, of the reaction with CoA was detected (data not shown), indicating that CoA is hydrolyzed by the enzyme to form 3',5'-ADP and 4'-phosphopantetheine. The $K_m$ value for the CoA ($12.4 \pm 5.4 \mu M$) of AtNUDX15a was similar to or lower than those of other CoA pyrophosphohydrolases, such as AtNUDX15 (12:0-CoA), and yeast Pcd1, 17) mouse Nudt7, 19) and C. elegans Y87G2A.14, 20) and higher than that of AtNUDX11 22) (Table 1). Previous studies have found that AtNUDX11 and AtNUDX15 are highly active toward various CoA derivatives. 23) To compare the enzymatic ability of AtNUDX11, 15, and 15a, kinetics parameters for various CoA derivatives were measured. The production and purification of the recombinant AtNUDX11 and 15 were carried out following previous reports. 22,23) AtNUDX11 showed higher affinity for malonyl-CoA (52.3 $\pm$ 8.9$\mu M$) than for CoA and the other CoA derivatives (Table 1). The $K_m$ value for the malonyl-

**Fig. 1.** Alternative Splicing Patterns of AtNUDX15 mRNAs and Putative Structures of AtNUDX15 Proteins.
A. Schematic diagrams of AtNUDX15 pre-mRNA and alternatively spliced mRNA variants of it (AtNUDX15 and 15a). The constitutive exons are shown as black boxes, the alternative exons as gray boxes, and the introns as lines. The 5'- and 3'- untranslated regions in each mRNA are shown as white boxes. Start (ATG) and stop (UAA and UGA) codons are indicated. Arrows (P-1 and P-2) indicate the locations of the primers in Fig. 2.

B. Alignment of the amino acid sequences of AtNUDX15, 15a, and 22, and mouse Nudt7a and C. elegans Y87G2A.14. Amino acids that are fully and highly conserved among CoA pyrophosphohydrolases are shown by black boxes. The bold-underlined, underlined, dotted-underlined, and double-underlined sequences are the Nudix motif, the UPF0035 motif, the mTP, and the PTS1 respectively.

**Fig. 2.** Changes in the Expression Levels of AtNUDX11, 15, and 15a in Various Plant Organs.
Semi-quantitative RT-PCR analysis of the AtNUDX11, 15, and 15a mRNAs in the rosette leaves, stems, cauline leaves, inflorescences, siliques, and roots of Arabidopsis plants grown in soil for 6 weeks under long-day conditions. PCR amplification was performed with 27–28 cycles of 95°C for 60 s, 55°C for 60 s, and 72°C for 60 s, followed by 72°C for 10 min. Aliquots of the products were analyzed on 1% agarose gel. Actin2 expression (18 cycles) was used as control in all experiments. Arrows indicate the bands of the AtNUDX15 and 15a mRNAs. The procedures are described in “Materials and Methods.”
Table 1. Comparison of Kinetic Parameters of the CoA Pyrophosphohydrolases in Arabidopsis, C. elegans, Mice, and S. cerevisiae

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (μmol/min/mg)</th>
<th>$k_{cat}$ (1/s)</th>
<th>$k_{cat}/K_m$ (1/s/M)</th>
<th>Reference</th>
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<td>CoA</td>
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<td></td>
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<td>AtNUDX11</td>
<td>27.8 ± 2.3</td>
<td>0.4 ± 0.01</td>
<td>0.18</td>
<td>6.5 x 10^4</td>
<td>23)</td>
</tr>
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<td>AtNUDX15</td>
<td>118.7 ± 3.4</td>
<td>1.4 ± 0.03</td>
<td>0.76</td>
<td>6.4 x 10^4</td>
<td>23)</td>
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<tr>
<td>AtNUDX15a</td>
<td>124.0 ± 5.4</td>
<td>1.5 ± 0.04</td>
<td>0.79</td>
<td>6.4 x 10^4</td>
<td>This study</td>
</tr>
<tr>
<td>C. elegans Y87G2A</td>
<td>220</td>
<td>—</td>
<td>13.8</td>
<td>6.3 x 10^4</td>
<td>20)</td>
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<tr>
<td>Mouse Nud7α</td>
<td>240</td>
<td>5.3</td>
<td>3.8</td>
<td>1.5 x 10^4</td>
<td>19)</td>
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<tr>
<td>S. cerevisiae PCD1</td>
<td>280</td>
<td>—</td>
<td>4.6</td>
<td>1.6 x 10^4</td>
<td>17)</td>
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<tr>
<td>Mouse RP2p</td>
<td>600</td>
<td>0.56</td>
<td>—</td>
<td>—</td>
<td>37)</td>
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<td>Succinyl-CoA</td>
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<tr>
<td>AtNUDX11</td>
<td>116.6 ± 12.8</td>
<td>10.3 ± 1.81</td>
<td>4.79</td>
<td>4.1 x 10^4</td>
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<tr>
<td>AtNUDX15</td>
<td>29.3 ± 9.5</td>
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<td>35.2 ± 10.5</td>
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<tr>
<td>AtNUDX11</td>
<td>52.3 ± 8.9</td>
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<td>2.90</td>
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<td>AtNUDX15</td>
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<td>1.4 x 10^4</td>
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<td>2.6 ± 0.65</td>
<td>1.38</td>
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<tr>
<td>Mouse Nud7α</td>
<td>330</td>
<td>4.1</td>
<td>3.0</td>
<td>9.0 x 10^4</td>
<td>19)</td>
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<td>Lauroyl-CoA (12-CoA)</td>
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<td>AtNUDX11</td>
<td>29.4 ± 4.6</td>
<td>11.4 ± 1.31</td>
<td>5.28</td>
<td>1.8 x 10^4</td>
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<td>2.8 ± 0.51</td>
<td>1.52</td>
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<tr>
<td>AtNUDX15a</td>
<td>17.8 ± 4.6</td>
<td>2.4 ± 0.68</td>
<td>1.28</td>
<td>7.2 x 10^4</td>
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<td>Mouse Nud7α</td>
<td>22</td>
<td>1.06</td>
<td>0.50</td>
<td>2.2 x 10^4</td>
<td>19)</td>
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<td>Mouse RP2p</td>
<td>80</td>
<td>0.2</td>
<td>—</td>
<td>—</td>
<td>37)</td>
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<td>Myristoyl-CoA (14-CoA)</td>
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<tr>
<td>AtNUDX11</td>
<td>23.7 ± 5.8</td>
<td>9.3 ± 1.78</td>
<td>4.30</td>
<td>1.8 x 10^5</td>
<td>This study</td>
</tr>
<tr>
<td>AtNUDX15</td>
<td>17.5 ± 2.8</td>
<td>1.7 ± 0.33</td>
<td>0.93</td>
<td>5.3 x 10^4</td>
<td>This study</td>
</tr>
<tr>
<td>AtNUDX15a</td>
<td>22.4 ± 4.9</td>
<td>2.0 ± 0.48</td>
<td>1.06</td>
<td>4.7 x 10^4</td>
<td>This study</td>
</tr>
<tr>
<td>Mouse Nud7α</td>
<td>34</td>
<td>0.41</td>
<td>0.19</td>
<td>8.1 x 10^5</td>
<td>19)</td>
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<tr>
<td>Oxidized-CoA (CoASSCoA)</td>
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<tr>
<td>AtNUDX11</td>
<td>180.9 ± 21.4</td>
<td>5.5 ± 0.63</td>
<td>2.56</td>
<td>1.4 x 10^4</td>
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</tr>
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<td>AtNUDX15</td>
<td>48.0 ± 3.4</td>
<td>3.2 ± 0.52</td>
<td>1.74</td>
<td>3.6 x 10^4</td>
<td>This study</td>
</tr>
<tr>
<td>AtNUDX15a</td>
<td>53.0 ± 6.9</td>
<td>3.4 ± 0.32</td>
<td>1.80</td>
<td>3.3 x 10^4</td>
<td>This study</td>
</tr>
<tr>
<td>Mouse RP2p</td>
<td>580</td>
<td>0.012</td>
<td>—</td>
<td>—</td>
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Subcellular localization of AtNUDX15 and 15a

We have reported that AtNUDX15 has N-terminal mTP and is localized to the mitochondrion;23) but the subcellular distribution of AtNUDX11 and AtNUDX15a remains unclear. As described above, AtNUDX15a has not only mTP at the N-terminus but also PTS1 at the C-terminal end. To confirm the subcellular distribution of these enzymes, full-length AtNUDX11, 15, and 15a cDNAs were fused in-frame with GFP at the N- or the C-terminus and then introduced into tobacco (Nicotiana tabacum) BY-2 cells. The fluorescence of the GFP-fused proteins in the transgenic cells was monitored by...
confocal microscopy. Fluorescence of AtNUDX11 fused to GFP at its C-terminus (AtNUDX11c-GFP) was detected in the cytosol (Fig. 4). The fluorescence of AtNUDX15a fused to GFP at the C-terminus (AtNUDX15ac-GFP) was co-localized with mitochondria stained with MitoTracker Orange, similarly to that of AtNUDX15 fused to GFP at C-terminus (AtNUDX15c-GFP), as reported previously.23) The fluorescence of AtNUDX15a fused to GFP at N-terminus (AtNUDX15an-GFP) showed dot-like patterns in the cytoplasmic area distinct from mitochondria, probably due to their peroxisomal localization,44,49,50) whereas that of AtNUDX15 fused to GFP at N-terminus (AtNUDX15n-GFP) was detected only in the cytosol (Fig. 4). These results suggest that the actions of the N-terminal mTP and C-terminal PST1 of AtNUDX15a predominates, the subcellular distribution of AtNUDX15 and AtNUDX15a fused to GFP via its internal regions (AtNUDX15i-GFP and AtNUDX15ai-GFP respectively) was analyzed. Fluorescence of AtNUDX15ai-GFP was detected in mitochondria as well as that of AtNUDX15i-GFP (Fig. 4). This indicates that the N-terminal mTP of AtNUDX15a predominates and hence that the protein is localized to the mitochondria. This result is similar to the case of rat serine:pyruvate aminotransferase (SPT), in that the C-terminal PTS1 of SPT has been reported to be inhibited by the N-terminal mTP.51)

Next, we confirmed the mitochondrial localization of AtNUDX15 and 15a in Arabidopsis plants. First, an anti-AtNUDX15 polyclonal antibody was generated using recombinant AtNUDX15. In immunoblot analyses, the polyclonal antibody recognized the recombinant protein in a range from 5 ng to 1 μg, and cross-reacted with AtNUDX11 and 15a (Fig. 5). In addition, it cross-reacted with the recombinant AtNUDX22 protein, which is highly homologous to AtNUDX15 (Fig. 5), although the protein had no activity toward any substrates.23)

Using the antibody obtained, the AtNUDX15 and 15a proteins (deduced molecular weights 32.8 and 31.9 kDa respectively) were not be detected in the crude extract prepared from 2-week-old Arabidopsis leaves grown under normal conditions (data not shown). Hence we isolated organelles from leaves of 6-week-old plants (20 g fresh weight) using the method described by Reumann et al.44) Fractions isolated on a Percoll density gradient were examined for catalase, SDH, and NADPH-GAPDH activities, markers for peroxisomes, mitochondria, and chloroplasts respectively. The highest levels of catalase, SDH, and NADPH-GAPDH activities were detected in fractions 2, 8, and 11 respectively (Fig. 6A). As in the case of SDH, the highest level of the CoA pyrophosphohydrolase activity was detected in fraction 8, although activities were also detected in the low-density fractions, including the chloroplast frac-

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**Fig. 4.** Subcellular Localization of AtNUDX11, 15, and 15a.
Confocal images of Tobacco BY-2 cells expressing the AtNUDX11, 15, and 15a proteins fused with GFP. Plasmids expressing AtNUDXs fused with GFP were introduced into Tobacco BY-2. Green fluorescence signals (a–g) of GFP and red fluorescence signals of mitochondria stained with MitoTracker (h–n) were detected with a laser confocal microscope (Bio-Rad, Hercules, CA). Merged images are shown in o–u. Scale bars are 10 μm. The procedures are described in the "Materials and Methods."
tions, probably due to contamination of the cytosolic AtNUDX11 protein in those fractions. However, on immunoblot analysis, AtNUDX15 and 15a were not detected in any fractions. These results suggest that the levels of AtNUDX15 and 15a in the leaves of Arabidopsis plants are extremely low or else undetectable based on post-translational protein modification.

Hence we generated transgenic Arabidopsis plants overexpressing AtNUDX15 and 15a under the control of the cauliflower mosaic virus 35S promoter (Pro35S:AtNUDX15 and Pro35S:AtNUDX15a respectively). There was no difference in growth or morphology between the control and transgenic plants under normal conditions. The levels of the AtNUDX15 and 15a mRNAs were higher in the T3 generation of the Pro35S:AtNUDX15 and 15a plants as compared with the control plants (Fig. 7A). Immunoblotting detected the AtNUDX15 protein in extracts prepared from the leaves of the Pro35S:AtNUDX15-1-1, -1-2, -10-1, and -10-2 plants (Fig. 7B). Similarly, the Pro35S:AtNUDX15a-2-1, -2-2, -3-1, and -3-2 plants accumulated the AtNUDX15a protein. The pyrophosphohydrolase activities toward CoA in the Pro35S:AtNUDX15 and Pro35S:AtNUDX15a plants were approximately 1.4- to 2.4-fold and 1.1- to 1.3-fold respectively higher than the levels in the wild-type plants (Fig. 7C). To confirm subcellular distribution of these enzymes, we isolated organelles from the Pro35S:AtNUDX15 and Pro35S:AtNUDX15a plants. Judging from the activities of catalase and SDH, fractions 4 and 9 isolated from the Pro35S:AtNUDX15a plants contained peroxisomes and mitochondria respectively (Fig. 6C). The highest level of CoA pyrophosphohydrolase activity was detected in the mitochondrial fraction. On immunoblot analysis, the

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Fig. 6. Subcellular Distribution of AtNUDX15 and 15a in Arabidopsis Plants.
Extracts prepared from wild-type (A), Pro35S:AtNUDX15-1-2 (B), and Pro35S:AtNUDX15a-2-1 (C) plants were fractionated by a discontinuous Percoll and sucrose gradient (15–38%, 0–36%). The distribution of CoA pyrophosphohydrolase activity and marker activities on the Percoll density gradient is shown. Catalase, SDH, and NADPH-GAPDH were markers of peroxisomes, mitochondria, and chloroplasts respectively. Fraction 1 represents the bottom of the gradient. Aliquots of the fractions (15 µL of each) were subjected to immunoblot analysis using the anti-AtNUDX15 polyclonal antibody.
AtNUDX15a protein was detected in the mitochondrial fraction. Similarly, AtNUDX15 was detected only in the mitochondrial fraction isolated from the Pro35S:AtNUDX15 plants (Fig. 6B). These results clearly indicate that AtNUDX15a as well as AtNUDX15 is localized exclusively to the mitochondria.

Conclusion

CoA and its derivatives depend on the metabolic state, and are compartmentalized in the cytosol, mitochondria, and peroxisomes, suggesting the importance of their own pool and of mechanism to tightly controling their levels in each organelle. In this study we characterized the molecular and enzymatic properties of Arabidopsis CoA pyrophosphohydrolases AtNUDX11, 15, and 15a. The cytosolic pool of malonyl-CoA contributes to various pathways, including fatty acid elongation and flavonoid biosynthesis, whereas the plastidic pool contributes to de novo fatty acid synthesis.52 Hence it appears likely that the actions of cytosolic AtNUDX11 impact many cellular processes in various organs, especially in the inflorescences. In addition, AtNUDX11 might contribute to the regulation of CoA biosynthesis by acting on the degradation of malonyl-CoA’s, since the activity of a key enzyme in CoA biosynthesis, PANK, is inhibited by malonyl-CoA, but not by CoA or acetyl-CoA.53 Analysis of the expression and subcellular distribution of AtNUDX15 and 15a suggested that the two enzymes act similarly in the mitochondria of various plant organs, while the latter has a specific function in inflorescences. In addition, it is possible that AtNUDX15 and 15a impact the regulation of the TCA cycle by the hydrolysis of succinyl-CoA, a key molecule in the cycle.54

The actions of CoA pyrophosphohydrolases toward free CoA or CoA derivatives produce 3'-0 ADP and (acyl-) 4'-phosphopantetheine. The fate of the products is unknown, but 4'-phosphopantetheine might be exported from the mitochondria to the cytosol and might be reused in the synthesis of CoA by PPAT. Although more studies are required, our results provide a starting-point in uncovering the importance of the degradation of CoA and its derivatives by CoA pyrophosphatehydrolases in plant cellular processes.

Acknowledgment

This work was supported by a Grant-in-Aid for Scientific Research (A) (S. S: 22248042) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, and by the Strategic Project to Support the Formation of Research Bases at Private Universities: A Matching Fund Subsidy from MEXT, 2011–2015 (S1101035).
References


