**Arabidopsis** Molybdopterin Biosynthesis Protein Cnx5 Collaborates with the Ubiquitin-like Protein Urm11 in the Thio-modification of tRNA

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† This article contains supplemental Materials and Methods, Figs. S1–S6, and Tables 1–5.

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**Background:** The plant proteins responsible for tRNA thio-modification are unclear.

**Results:** Ubiquitin-like protein Urm11 and the molybdopterin biosynthesis protein Cnx5 participate together in tRNA thio-modification in **Arabidopsis**.

**Conclusion:** Cnx5 is a ubiquitin-activating enzyme-like protein that functions in two different metabolic processes with two distinct ubiquitin-like partners.

**Significance:** Cnx5-Urm11 ortholog pair-mediated thio-modification of tRNA is ubiquitous in most eukaryotes, including plants.

The thio-modification of tRNA that occurs in virtually all organisms affects the accuracy and efficiency of protein translation and is therefore biologically important. However, the molecular mechanism responsible for this tRNA modification in plants is largely unclear. We demonstrate here that **Arabidopsis** sulfurtransferase Cnx5, a ubiquitin-activating enzyme-like (Ubl) protein involved in molybdopterin (MPT) biosynthesis, is strictly required for the thio-modification of cytosolic tRNAs *in vivo*. A previously uncharacterized ubiquitin-like (Ubl) protein Urm11 is also essential for tRNA thio-modification in **Arabidopsis**. When expressed in *Saccharomyces cerevisiae*, Cnx5 and Urm11 can substitute for the corresponding yeast orthologs ScUba4 and ScUrm1, respectively, in the thio-modification of yeast cytosolic tRNAs. However, another Ubl protein, Cnx7 of **Arabidopsis**, which is involved in MPT biosynthesis in conjunction with Cnx5, cannot replace yeast ScUrm1. Interestingly, the expression of a mutant form of Cnx7 in which the carboxyl-terminal six amino acids are substituted by those of Urm11 can significantly restore the thio-modification of tRNAs in the yeast urm1Δ mutant. These findings suggest that in **Arabidopsis** the common UBA protein Cnx5 collaborates with two functionally differentiated Ubl proteins, Urm11 and Cnx7, in the thio-modification of tRNA and MPT biosynthesis, respectively. Phylogenetic analysis revealed that although most eukaryotes contained a Cnx5-Urm11 ortholog pair and the tRNA thio-modification some fungi, including *S. cerevisiae*, had lost the Cnx7 ortholog and the ability to synthesize the molybdenum cofactor.

A wide variety of post-transcriptional tRNA modifications have been identified in virtually all organisms from bacteria to animals and plants (1). Among these modifications, the thio-modification of the first anticodon uridine of tRNA\(^{\text{Lys}}_{UUU}\) and tRNA\(^{\text{Glu}}_{UUC}\) (the wobble uridine) is physiologically important because it affects proper codon recognition (2). The molecular mechanisms for tRNA thio-modification in eukaryotic cells have primarily been studied using yeast, especially *Saccharomyces cerevisiae*. In the yeast *S. cerevisiae*, cytosolic and mitochondrial tRNAs are thio-modified by partially overlapping multi-step mechanisms in which several cytosolic and mitochondrial proteins participate (3–7). A common initial step for these thio-modifications involves the mitochondrial cysteine desulfurase Nfs1, which cleaves sulfur from its substrate cysteine to form the enzyme-bound cysteinyl persulfide intermediate (3). Sulfur is then incorporated into either cytosolic or mitochondrial substrate tRNAs through distinct sulfur-transferring systems (4). Interestingly, the sulfur-transferring system for cytosolic tRNAs involves some essential proteins that are required for the maturation of cytosolic iron-sulfur proteins (4).

In *S. cerevisiae*, the ubiquitin-like (Ubl)\(^2\) protein ScUrm1 and the ubiquitin-activating enzyme-like (UBA) protein ScUba4, which are both located in the cytosol, are also involved in the thio-modification of cytosolic tRNAs (6–10). Similar to the common activation mechanism of various Ubl proteins that is used by UBA proteins, the C-terminal glycine of ScUrm1 is first adenylated by the N-terminal MoeB-like domain of ScUba4 and then further modified to a thiocarboxylate by the rhodanese (RHD)-like domain of ScUrm1 (11, 12); the sulfur is donated from an undefined direct donor. Next, the thiocarboxylated Urm1 is thought to provide sulfur for the thio-modification of the first anticodon uridine of tRNA and for the mitochondrial cysteine desulfurase Nfs1, which cleaves sulfur from its substrate cysteine to form the enzyme-bound cysteinyl persulfide intermediate (3). Sulfur is then incorporated into either cytosolic or mitochondrial substrate tRNAs through distinct sulfur-transferring systems (4). Interestingly, the sulfur-transferring system for cytosolic tRNAs involves some essential proteins that are required for the maturation of cytosolic iron-sulfur proteins (4).

The abbreviations used are: Ubl, ubiquitin-like; UBA, ubiquitin-activating enzyme-like; MPT, molybdopterin; RHD, rhodanese; MoCo, molybdenum cofactor; At, *A. thaliana*; Sc, *S. cerevisiae*; Hs, *Homo sapiens*; At-cyK, *A. thaliana* cytosolic tRNA\(^{\text{Lys}}_{UUU}\); At-cyE, *A. thaliana* cytosolic tRNA\(^{\text{Glu}}_{UUC}\); Sc-cyK, *S. cerevisiae* cytosolic tRNA\(^{\text{Lys}}_{UUU}\); Sc-cyE, *S. cerevisiae* cytosolic tRNA\(^{\text{Glu}}_{UUC}\); SAMP, small archaeal modifier protein.
ificiation of cytosolic tRNAs. This step is presumably aided by additional components, such as Ncs6 (Tuc1) and Ncs2 (Tuc2), the exact roles of which are still unclear (5, 6, 9).

Despite the recent identification of some orthologous proteins in other eukaryotes, including mammalian cells (8, 9, 13), whether plants utilize similar mechanisms for tRNA thio-modification remains largely unclear and has not been well investigated. Moreover, the thio-modification of any specific cytosolic tRNA molecule in plant cells has not yet been demonstrated. Intriguingly, the reaction steps in tRNA thio-modification that involve the previously mentioned Urm1 and Uba4 proteins are mechanistically related to the sulfur transfer reactions that are found in the biosynthesis of the molybdenum cofactor (MoCo) in *Escherichia coli*. These reactions require the Ubl protein MoaD and the UBA protein MoeB (14). The MoeB-catalyzed formation of thio-carboxylate at the C-terminal glycine of MoaD is necessary to transfer the sulfur atom to the cyclic pyranopterin monophosphate to form molybdopterin (MPT), which is the precursor of MoCo (15, 16). Similar MoCo biosynthetic pathways have been identified in plants and animals (13, 17–19). In plants, Cnx (cofactor for nitrate reductase and xanthine dehydrogenase) proteins have been shown to participate in multistep reactions (17). The MPT synthase sulfurtransferase Cnx5, which is a UBA protein, transfers sulfur to Cnx7, which is a Ubl protein, and forms a complex with Cnx6 (18) after which the sulfur is incorporated into cyclic pyranopterin monophosphate to form MPT. Orthologs of Cnx5, Cnx7, and Cnx6 are also present in humans and are termed MOCS3, MOCS2A, and MOCS2B (for MoCo synthesis), respectively (19). *S. cerevisiae* has no such MoCo biosynthetic enzymes because this organism lacks the MoCo biosynthesis trait due to the absence of any MoCo-dependent enzymes.

In *Arabidopsis thaliana*, AtCnx5 is the only protein that has been identified in the entire genome that shows significant sequence similarity to ScUba4, which is the UBA protein involved in the thio-modification of yeast cytosolic tRNAs. Herein, we demonstrate that AtCnx5, which is involved in MoCo synthesis, is also essential for the wobble thio-modification of cytosolic tRNAs in *Arabidopsis*, and this protein can fully complement the yeast *uba4Δ* mutant. In addition, although AtCnx7, a well known partner Ubl for AtCnx5 in MoCo synthesis, cannot replace ScUrm1 in yeast *urm1Δ* cells, another Ubl protein, here termed AtUrm1, can substitute for ScUrm1 and is an essential contributor to cytosolic tRNA thio-modification in *Arabidopsis*. Therefore, in plant cells, a common UBA protein is used for both the tRNA thio-modification and MoCo biosynthetic pathways in conjunction with a distinct type of partner Ubl protein for each pathway.

**EXPERIMENTAL PROCEDURES**

**Plant and Yeast Strains, Plasmids, Media, and Growth Conditions**—The *A. thaliana* and *S. cerevisiae* strains and plasmids used in complementation analyses are summarized in supplemental Table 1. Oligonucleotide primers and probes are listed in supplemental Table 2. *A. thaliana* T-DNA insertion mutants *cnx5-1* (FLAG_116G03), *cnx5-2* (SALK_039229), *urm11-1* (SALK_024513), *urm11-2* (SAIL_198D05), *urm12-1* (SALK_061839), and *urm12-2* (SALK_070672) were obtained from the Institut National de la Recherche Agronomique and from the Salk Institute Genomic Analysis Laboratory. *Arabidopsis* ecotype Columbia-0 was used as the wild type. *Arabidopsis* plants were grown on Murashige and Skoog medium (Wako) containing 1% Phytalgel (Sigma-Aldrich) and 3% sucrose or on soil under 16-h light/8-h dark cycles at 23 °C. The T-DNA insertions were confirmed using genomic PCR.

The *S. cerevisiae* strains *urm1Δ* and *uba4Δ* were purchased from Thermo Scientific. The yeast cells were grown in synthetic complete medium that was supplemented with 2% galactose and 3% glycerol (SGGly-His) but lacked histidine for GAL110 promoter-dependent production of the plasmid-borne proteins. The yeast strains were grown at 30 °C until they reached log phase, and serial 5-fold dilutions of cell cultures were spotted onto synthetic solid medium and incubated for 5 days at 30 °C. The complete open reading frames of *AtCNX5*, *AtURM11*, *AtUIRM12*, and *AtCNX7* were amplified from the cDNA library of wild-type *Arabidopsis* using PCR and cloned into the pESC-HIS vector (Stratagene). The cDNA fragments were further modified to encode various mutants. *E. coli* strain JM109 was used for routine recombinant experiments and grown at 37 °C in Luria-Bertani medium.

**tRNA Extraction and Gel Retardation Analysis of the Thio-modification of tRNA**—The total tRNAs of the yeast cells were prepared as described previously (3). The plant seedlings were grown on Murashige and Skoog plates for 3 weeks, and tRNAs were extracted using the PureLink miRNA Isolation kit (Invitrogen). The gel retardation analyses of the thio-modification of the yeast and plant tRNAs were performed as described previously (3). [(N-Acryloylamino)phenyl]mercuric chloride was synthesized according to Igloi (21). The thio-modified uridine of tRNA was verified through the retardation of electrophoretic mobility on polyacrylamide gels containing [(N-acryloylamino)phenyl]mercuric chloride and detected using hybridization with a DNA probe specific for each tRNA. All thio-modification assays were performed in triplicate, and the means and S.D. values are shown as described previously (3).

**Database Searches and Protein Sequence Alignments**—Homologs of Urm1, Cnx5, and Cnx7 were identified from the protein and nucleotide databases of various organisms provided by the NCBI (National Center for Biotechnology Information) using BLASTp and tBLASTn searches (supplemental Table 3). The Conserved Domain Search program at NCBI was also used to detect structural and functional domains. The amino acid sequences were aligned using the ClustalW2 program (European Bioinformatics Institute) and modified manually. To confirm the orthologous relationships among the sequences for each protein alignment, phylogenetic trees were reconstructed using the maximum likelihood method.

**RESULTS**

AtCnx5 Is Required for the Thio-modification of Nuclearily Encoded Cytosolic tRNA<sup>lys</sup><sub>thm</sub> and tRNA<sup>Glu</sup><sub>thm</sub> in *Arabidopsis*—The protein that is encoded by *Arabidopsis* gene *At5g55130* shares more than 76% overall sequence identity with *Nicotiana plumbaginifolia* Cnx5, which has been demonstrated to be involved in MoCo biosynthesis (22), and 33.8% identity (69.2%...
similarity) with the human ortholog MOCS3 (HsMOCS3). Therefore, this Arabidopsis protein was annotated as AtCnx5 (Fig. 1A). Impairment in the synthesis of MoCo has been demonstrated in the Arabidopsis cnx5 mutant (formerly called sir1 (23)) plant (24). AtCnx5 also shows significant sequence similarity with ScUba4 (36.6% identity and 72.8% similarity), and we could not identify any other homologous protein encoded by the Arabidopsis genome. AtCnx5, HsMOCS3, and ScUba4 share a characteristic two-domain structure that consists of an N-terminal MoeB-like domain (Fig. 1A, gray line) followed by a C-terminal RHD-like domain (Fig. 1A, black line). However, whether AtCnx5 or any Cnx5 ortholog in other plants is

**FIGURE 1.** The Arabidopsis cnx5-1 mutant exhibits severe growth defects and impaired thio-modification of cytosolic tRNAs. Three eukaryotic sulfurtransferase protein sequences from Arabidopsis (AtCnx5), S. cerevisiae (ScUba4), and H. sapiens (HsMOCS3) were compared, and the bacterial E. coli sequence (EcMoeB) was used as a reference (A). Sequences that are conserved among at least two of the three eukaryotic proteins are highlighted in magenta. A conserved domain NCBI search revealed a conserved domain shared by the ThiF_MoeB_HesA family of E1-like enzymes that are involved in MoCo and thiamine biosynthesis (cd00757, gray line) and the RHD-like protein family (cd01526, black line). The P-loop motif and the CXXC putative metal-binding sites are indicated above the sequences, and the consensus motif found in RHD-like proteins is boxed. The AtCNX5 gene structure and the position of the T-DNA insertion in cnx5-1 are shown in B. The positions of the primers used for PCR genotyping are also indicated. The progeny of the heterozygous cnx5-1/H11001 line were grown for 21 days, and PCR genotyping of these progeny identified the homozygous CNX5 (a), heterozygous cnx5-1 (b), and homozygous cnx5-1 mutant (c) plants in C. Representative seedlings from these different genotypes (the bars indicate 1 cm) and the results from the genomic PCR are shown. In D, the gel retardation assay revealed that AtCnx5 was essential for the thio-modification of cytosolic tRNA. Representative images of the thio-modified and non-thio-modified tRNAs hybridized with the DNA probes At-cyK and At-cyE are shown in the top panel, and the thio-modified tRNAs are quantified in the bottom panel. The error bars represent the standard deviation.
involved in the thio-modification of tRNAs has not yet been investigated.

To examine this question, we analyzed a T-DNA insertion mutant of the AtCNX5 gene (hereafter called cnx5-1) that originated from the FLAGdb/FST collection line FLAG_116G03 (Fig. 1B). When the progeny of the heterozygous cnx5-1/+ plant were grown on sucrose-containing medium, a proportion of the seedlings exhibited a striking dwarf phenotype with severe growth defects (19.0%; 205 dwarf plants of 1078 seedlings) (Fig. 1C). They had slightly green and morphologically aberrant leaves and did not grow well. The seedlings did not produce a stalk or flower, and they were therefore sterile. Genomic PCR genotyping revealed that all of the dwarf individuals were homozygous cnx5-1 mutant. However, the heterozygous cnx5-1/+ mutants did not show any growth defects compared with the homozygous CNX5(+/+) plants. The observed proportion of homozygous dwarf plants was clearly less than the expected value of 25% for normal inheritance, which suggests that the homozygotes have reduced viability at an early developmental stage, such as seed formation or maturation.

The 3-week-old homozygous cnx5-1 mutants and CNX5 (+/+) plants were collected separately, and their total low molecular weight RNA was subjected to a gel retardation assay to analyze the accumulation of thio-modified cytosolic tRNA (Fig. 1D). In this Northern assay, the thio-modification status of any tRNA can be assessed through the retardation of the electrophoretic migration of its band, which is detected using a specific probe for each tRNA in an [(N-acryloylaminophenyl)mercuric chloride- containing gel. A significant fraction of the tRNAs from the wild-type plants showed retardation when specific probes corresponding to either A. thaliana cytosolic tRNA\textsubscript{UUC}\textsuperscript{Lys} (At-cyK) or A. thaliana cytosolic tRNA\textsubscript{UUC}\textsuperscript{Glu} (At-cyE) were used for detection; both of these tRNAs are encoded in the nuclear genome of Arabidopsis. These data indicate that a significant proportion of these specific cytosolic tRNAs are thio-modified in Arabidopsis. In contrast, no such retardation was observed when tRNAs from the homozygous cnx5-1 plants were analyzed. The impairment in the thio-modification of cytosolic tRNAs in the homozygous cnx5-1 mutant clearly indicates the critical contribution of AtCnx5 to the thio-modification of At-cyK and At-cyE tRNAs. This conclusion was further confirmed by a similar analysis using a subsequently identified additional allele named cnx5-2 (originated from SALK_039229 line) (supplemental Fig. S1). Both cnx5-1 and cnx5-2 alleles are considered to be null because of the absence of CNX5 transcripts as revealed by RT-PCR analysis (supplemental Materials and Methods) and (supplemental Fig. S1D). The homozygous cnx5-2 mutant exhibited a dwarf phenotype with severe growth defects similar to that seen for the cnx5-1 mutant.

AtCnx5 Can Functionally Replace ScUba4 for the Thio-modification of Cytosolic tRNAs in the Yeast uba4\Delta Mutant—We next examined the ability of AtCnx5 to complement the defective thio-modification of the cytosolic tRNAs in yeast uba4\Delta cells. As demonstrated previously (6), wobble thio-modifications of S. cerevisiae cytosolic tRNA\textsubscript{UUC}\textsuperscript{Lys} (Sc-cyK) and tRNA\textsubscript{UUC}\textsuperscript{Glu} (Sc-cyE) were completely abolished in uba4\Delta cells (Fig. 2A).
The ectopic expression of plasmid-borne AtCnx5 in uba4Δ mutants led to substantial restoration of tRNA thio-modification that was almost as efficient as ScUba4 (Fig. 2A). AtCnx5, ScUba4, and HsMOC3 possess the C-terminal RHD-like domain, but this RHD-like domain does not exist in E. coli or some other bacterial MoeB proteins. However, a mutant form of AtCnx5 that lacked the C-terminal RHD domain failed to restore the thio-modification of cytosolic tRNAs in the yeast uba4Δ mutant (Fig. 2A, lane 4). To confirm that this was not due to a low expression or rapid degradation of the RHD domain-lacking mutant, the N-terminally Strep-tagged derivatives of AtCnx5 (St-AtCnx5-full and St-AtCnx5-w/oRHD) were expressed in the uba4Δ mutants for immunological detection (supplemental Fig. S2A). Although expression levels of the two proteins were quite similar, only the full-length AtCnx5 derivative (St-AtCnx5-full) could restore the thio-modification of cytosolic tRNAs in the uba4Δ mutant. We also determined that for ScUba4 the C-terminal RHD-like domain was dispensable for tRNA thio-modification (supplemental Fig. S3). These results clearly indicate that the C-terminal RHD-like domain of this protein family is absolutely required for tRNA modification in the eukaryotic cytosol.

Some yeast mutants that display defects in cytosolic tRNA thio-modification are more susceptible to oxidative stress caused by thiol-oxidizing agents, such as diamide, or rapamycin treatment, which affects the target of rapamycin signaling pathway that is thought to regulate the balance between protein synthesis and protein degradation (9, 25–27). The yeast uba4Δ mutant also exhibited such growth defects on medium that contained diamide or rapamycin, and this defect was suppressed by the ectopic expression of the wild-type ScUba4 or AtCnx5 protein (Fig. 2B). Together, these data indicate that the Arabidopsis MPT biosynthesis protein Cnx5 fulfills the physiological roles of yeast Uba4 as an essential protein for the thio-modification of cytosolic tRNAs.

A Previously Uncharacterized Ubl Protein, AtUrm11, Is Required for the Thio-modification of Cytosolic tRNAs in Arabidopsis—Cnx7 (encoded by At4g10100 in Arabidopsis) is a partner Ubl protein of Cnx5 and is also essential for MPT synthesis in MoCo biogenesis. AtCnx5 is thought to catalyze the formation of thiocarboxylate at the C-terminal glycine of AtCnx7 via a sulfur transfer mechanism that likely resembles the transfer that occurs between ScUba4 and ScUrm1, the latter of which is the yeast Ubl protein required for cytosolic tRNA thio-modification and functions in cooperation with ScUba4 (19, 28). However, AtCnx7 shares only limited sequence identity with ScUrm1 (14%). In addition to AtCnx7, two previously uncharacterized Arabidopsis genes, AtURM11 (At2g45695) and AtURM12 (At3g61113), potentially encode orthologs of ScUrm1. AtUrm11 and AtUrm12 exhibit 87% sequence identity and 93% sequence similarity with each other, and they both show significant sequence identity to human Urm1 (HsUrm1) (55 and 54%, respectively) and ScUrm1 (37 and 38%, respectively) (Fig. 3A).

To examine the phenotypes of Arabidopsis knock-out mutants of AtURM11 and AtURM12, we obtained Salk T-DNA insertion mutant lines. SALK_024513 has a T-DNA insertion in the first intron of the AtURM11 gene, and SALK_061839 has a T-DNA insertion in the fourth exon of the AtURM12 gene (Fig. 3B). We verified the correct locations of these T-DNA insertions using genomic PCR. From the progeny of these lines, we identified homozygous T-DNA insertion mutants of AtURM11 and AtURM12 (hereafter named urm11-1 and urm12-1, respectively) (Fig. 3C). Unlike the cnx5-1 mutant, both of the urm11-1 and urm12-1 mutant phenotypes were indistinguishable from that of the wild-type Columbia-0 throughout development when they were grown on either sucrose-containing medium or soil (Fig. 3C).

Using the gel retardation assay described above, we analyzed whether the thio-modification of cytosolic tRNAs occurred normally in the urm11-1 and urm12-1 mutants. Although the urm11-1 mutant showed no observable growth defects, the thio-modification of At-cyK and At-cyE tRNAs was greatly impaired (Fig. 3D, lane 1). However, such thio-modification impairments of the cytosolic tRNAs were not observed in the urm12-1 mutants compared with the wild-type plants (Fig. 3D, lanes 2 and 3), which indicates that AtUrm11 critically contributes to the thio-modification of cytosolic tRNAs in Arabidopsis. This was further confirmed by similar analyses using two subsequently identified additional alleles named urm11-2 and urm12-2 (originated from SAIL_198D05 line and SALK_070672, respectively) of which only the former mutant was impaired in the thio-modification of cytosolic tRNAs (supplemental Fig. S4). RT-PCR analysis confirmed that both the urm12-1 and urm12-2 alleles as well as urm11-1 and urm11-2 alleles are considered to be null mutations because of the absence of corresponding transcripts (supplemental Materials and Methods) and (supplemental Fig. S4D). Thus, although we could detect transcripts for AtURM12 in urm11 mutants, the contribution of AtUrm12 in the cytosolic thio-modification of tRNAs is less clear.

Both AtUrm11 and AtUrm12 Can Partially Replace ScUrm1 in the Thio-modification of Cytosolic tRNAs in Yeast Cells—Next, we examined whether AtUrm11 and AtUrm12 could fulfill the functions of ScUrm1 when expressed in yeast urm1Δ mutant cells. We also examined whether AtCnx7 could at least partially replace the functions of ScUrm1 because the AtCnx7-interacting partner AtCnx5 could fully replace the function of ScUba4 in yeast cells as shown in Fig. 2. Therefore, AtUrm11, AtUrm12, and AtCnx7 were separately expressed in yeast urm1Δ cells. Because ScUrm1 is strictly required for the thio-modification of cytosolic tRNAs in S. cerevisiae (6, 9), the urm1Δ mutant did not contain any detectable thio-modification of cytosolic tRNAs (Fig. 4A, lane 7), and this defect was rescued through the ectopic expression of plasmid-borne ScUrm1 (Fig. 4A, lane 6). The expression of either AtUrm11 or AtUrm12 in the urm1Δ mutant significantly restored the levels of thio-modification of both cytosolic tRNAs (Fig. 4A, lanes 2 and 3), whereas AtCnx7 did not lead to such a recovery (Fig. 4A, lane 1) even though the protein level of AtCnx7 reached a level comparable with that of yeast ScUrm1 (supplemental Fig. S2C). The levels of thio-modified tRNAs were not markedly different between the urm1Δ mutant cells that expressed either AtUrm11 or AtUrm12. However, compared with the recovery of thio-modified tRNAs in response to ScUrm1 expression, tRNA thio-modification in response to AtUrm1 and AtUrm12...
was less efficient. These results indicate that these Arabidopsis homologs can partially replace ScUrm1 in cytosolic tRNA thio-modification in yeast cells. Nevertheless, it is obvious that the AtUrm11 and AtUrm12 proteins are specifically involved in recovery of the thio-modification of cytosolic tRNAs in the urm1Δ mutant. The expression of mutant forms of the AtUrm11 and AtUrm12 proteins lacking the C-terminal Gly-Gly sequence completely abolished the restoration of thio-modification (Fig. 4, lanes 4 and 5). This result has been observed previously with a similar mutation in ScUrm1 (6).

To exclude the possibility that the observed inability of AtUrm11 and AtUrm12 mutants lacking the C-terminal Gly-Gly sequence to restore the thio-modification of cytosolic tRNAs was due to their low expression or instability, similar analyses were performed for the N-terminally Strep-tagged derivatives (supplemental Fig. S2B). Although the expression levels of the C-terminal Gly-Gly-lacking mutants of AtUrm11 and AtUrm12 (St-AtUrm11-w/oGG and St-AtUrm12-w/oGG, respectively) were quite similar to those of the corresponding full-length proteins and the Cnx7/MOCS2A-type or MoaD-type Ubl proteins are shown in orange, whereas those that are uniquely conserved between the latter two types are shown in gray. In B, the gene structures of AtURM11 and AtURM12 and the positions of the T-DNA insertions in the urm11-1 and urm12-1 mutants are shown. The homozygous urm11-1 and urm12-1 mutant seedlings showed no phenotypic changes compared with the wild-type (Columbia-0 (Col-0)) seedlings (all bars indicate 1 cm) (C). In D, the gel retardation assay against At-cyK and At-cyE was performed as shown in Fig. 1, and thio-modification of the cytosolic tRNAs was severely impaired in the urm11-1 mutant (lane 1) but not in urm12-1 (lane 2) or wild type (lane 3). The error bars represent the standard deviation.

**FIGURE 3.** AtUrm11 but not AtUrm12 is required for the thio-modification of cytosolic tRNAs in Arabidopsis. In A, the eukaryotic Urm11/Urm1-type Ubl proteins from Arabidopsis (AtUrm11 and AtUrm12) were aligned with orthologs in the yeast *S. cerevisiae* (ScUrm1) and human (HsUrm1). They were further compared with another type of eukaryotic Ubl protein (Cnx7/MOCS2A-type), AtCnx7 and its human ortholog (HsMOCS2A), and the bacterial *E. coli* MoaD sequence (EcMoaD) was used as a reference. The amino acid residues that are conserved between the Urm11/Urm1-type Ubl proteins and the Cnx7/MOCS2A-type or MoaD-type Ubl proteins are shown in orange, whereas those that are uniquely conserved between the latter two types are shown in gray. In B, the gene structures of AtURM11 and AtURM12 and the positions of the T-DNA insertions in the urm11-1 and urm12-1 mutants are shown. The homozygous urm11-1 and urm12-1 mutant seedlings showed no phenotypic changes compared with the wild-type (Columbia-0 (Col-0)) seedlings (all bars indicate 1 cm) (C). In D, the gel retardation assay against At-cyK and At-cyE was performed as shown in Fig. 1, and thio-modification of the cytosolic tRNAs was severely impaired in the urm11-1 mutant (lane 1) but not in urm12-1 (lane 2) or wild type (lane 3). The error bars represent the standard deviation.
Thio-modification of Plant tRNA

We show here that the plant Arabidopsis Cnx5, which is a UBA protein involved in the MPT synthesis in MoCo biogenesis, also participates in the thio-modification of nuclearly encoded cytosolic tRNA\(^{\text{cys}}\), and tRNA\(^{\text{Glu}}\) in vivo and that this dual function of AtCnx5 is dependent upon two functionally distinct Ubl partner proteins: AtUrm11 is critical for the thio-modification of tRNA, and AtCnx7 is required for MoCo biosynthesis (Fig. 5). The six C-terminal amino acids of the two types of Ubl proteins, which are highly conserved within each type but clearly distinct between the two types, appear to be the major determinants of the fate of bound sulfur, which can be used for either the thio-modification of cytosolic tRNAs or MPT biosynthesis.

DISCUSSION

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To explore the relationship between the phylogenetic distributions of the MoCo utilization trait and the UBA-Ubl protein

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pair(s) that have been studied in the present work, we used tBLASTn to search various eukaryotic genome databases in NCBI for orthologous genes of the Cnx5/MOCS3/Uba4-type sulfur transfer UBA proteins, the MPT synthesis-related Ubl proteins, and the tRNA thio-modification-related Ubl proteins (supplemental Materials and Methods). We found that the sulfur transfer UBA proteins and the tRNA thio-modification-related Ubl proteins were ubiquitously present in virtually all of the eukaryotic phyla (Table 1). In contrast, the MPT synthesis-related Ubl proteins were widely distributed among plants, vertebrates, and some fungi, but they were not present in parasitic protozoa and certain fungi, including S. cerevisiae (Table 1). For example, various eukaryotic parasites belonging to Kinetoplastida, Apicomplexa, Parabasalidea, Entamoebidae, and Diplomonadida possessed the sulfur transfer UBA proteins and the tRNA thio-modification-related Ubl proteins (supplemental Materials and Methods). We found that the sulfur transfer UBA proteins and the tRNA thio-modification-related Ubl proteins were ubiquitously present in virtually all of the eukaryotic phyla (Table 1). In contrast, the MPT synthesis-related Ubl proteins were widely distributed among plants, vertebrates, and some fungi, but they were not present in parasitic protozoa and certain fungi, including S. cerevisiae (Table 1). For example, various eukaryotic parasites belonging to Kinetoplastida, Apicomplexa, Parabasalidea, Entamoebidae, and Diplomonadida possessed the sulfur transfer UBA proteins and the tRNA thio-modification-related Ubl proteins, but they did not encode the MPT synthesis-related Ubl proteins. In Ascomycota, fungi belonging to Saccharomycotina, including S. cerevisiae, possessed the sulfur transfer UBA proteins and the tRNA thio-modification-related Ubl proteins, but they did not encode the MPT synthesis-related Ubl proteins. As anticipated, Schizosaccharomyces, which is the most closely related group of fungi to Saccharomycotina, possessed the former two types of proteins but did not possess the MPT synthesis-related Ubl proteins. Interestingly, Pezizomycotina, which includes Aspergillus flavus and Neurospora crassa and is another close sister group to Saccharomycotina in Ascomycota, retained all three types of proteins. This phylogenetic distribution of the MPT synthesis-related Ubl proteins correlates well with the MoCo utilization trait and the MoCo-dependent enzymes (29). In other words, the organisms that have lost the use of MoCo-dependent enzymes have also lost the MPT synthesis-related Ubl proteins.

More importantly, virtually all eukaryotes appear to retain the Cnx5/MOCS3/Uba4-type sulfur transfer UBA proteins together with the Urm11/Urm1-type tRNA thio-modification-related Ubl proteins for the thio-modification of cytosolic tRNAs (Table 1 and supplemental Fig. S6), and this result suggests the fundamental physiological importance of the wobble thio-modification of certain cytosolic tRNAs. The conservation of both types of proteins seems to be a paradox because the absence of cytosolic thio-modification of tRNAs does not cause severe growth defects in S. cerevisiae or Arabidopsis. Indeed, under normal growth conditions, the yeast urm1Δ and uba4Δ mutants were viable and grew normally (Figs. 2B and 4B). Similarly, we demonstrated here that the Arabidopsis urm11-1 T-DNA knock-out mutant did not exhibit any visible phenotypic changes, although the mutant lacked thio-modified cytosolic tRNAs (Fig. 3C). This result shows a marked difference from the severe growth defects and lethal phenotype of the Arabidopsis cnx5-1 mutant (Fig. 1C). Because both mutants lacked thio-modified cytosolic tRNAs (Figs. 1D and 3D), the severe phenotype of the cnx5-1 mutant may be reasonably attributed to the MoCo biosynthesis defect in the mutant rather than the defective thio-modification of tRNAs. Nevertheless, the apparent evolutionary pairwise conservation of the sulfur transfer UBA proteins and the tRNA thio-modification-related Ubl proteins likely indicates their necessity under certain specific circumstances, although they are dispensable under normal laboratory growth conditions. In this context, it is noteworthy that yeast mutants, including the urm1Δ and uba4Δ mutants, that exhibit the impaired thio-modification of cytosolic tRNAs are susceptible to various severe growth conditions, such as oxidative stress and amino acid starvation (25–27, 30). The presence or absence of the wobble thio-modification of cytosolic tRNAs affects the decoding capacity of NAA and

FIGURE 5. Sulfur delivery for the thio-modification of tRNA and for MPT biosynthesis in plant and yeast. In the plant A. thaliana (left), the sulfur transfer UBA protein AtCnx5 provides sulfur to two functionally different Ubl proteins: AtUrm11 for the thio-modification of tRNAs and AtCnx7 for MPT biosynthesis. However, in the yeast S. cerevisiae (right), the sulfur transfer UBA protein ScUba4 provides sulfur (red filled circle) to its partner Urm11/Urm1-type Ubl protein ScUrm1 for the thio-modification of cytosolic tRNAs, and no such UBA-Ubl pair is found for MPT biosynthesis or MoCo biosynthesis. The physiological function of AtUrm12 is less clear.
| Phylum                        | Organism                  | MPT synthesis-related Ubl protein | the sulfur-transfer UBA protein | tRNA thio-modification-related Ubl protein |
|------------------------------|---------------------------|----------------------------------|--------------------------------|------------------------------------------|
| Plants                       | Arabidopsis thaliana      | +                                | +                              | +                                        |
|                              | Populus trichocarpa       | +                                | +                              | +                                        |
|                              | Oryza sativa              | +                                | +                              | +                                        |
| Vertebrates                  | Bos taurus                | +                                | +                              | +                                        |
|                              | Pan troglodytes           | +                                | +                              | +                                        |
|                              | Homo sapiens              | +                                | +                              | +                                        |
|                              | Canis familiaris          | +                                | +                              | +                                        |
|                              | Equus caballus            | +                                | +                              | +                                        |
|                              | Mus musculus              | +                                | +                              | +                                        |
|                              | Xenopus tropicalis        | +                                | +                              | +                                        |
|                              | Danio rerio               | +                                | +                              | +                                        |
|                              | Tetraodon nigroviridis    | +                                | +                              | +                                        |
| Insects                      | Drosophila melanogaster   | +                                | +                              | +                                        |
|                              | Bombyx mori               | +                                | +                              | +                                        |
|                              | Anopheles gambiae         | +                                | +                              | +                                        |
| Dictyostelida                | Dictyostelium discoideum  | +                                | +                              | +                                        |
| MoCo-utilizing Fungi         | Aspergillus flavus        | +                                | +                              | +                                        |
|                              | Neurospora crassa         | +                                | +                              | +                                        |
|                              | Gibberella zeae           | +                                | +                              | +                                        |
|                              | Magnaporthe grisea        | +                                | +                              | +                                        |
|                              | Botryotinia fuckeliana    | +                                | +                              | +                                        |
| MoCo-Non-utilizing Fungi     | Debaryomyces hansenii     | −                                | +                              | +                                        |
|                              | Candida albicans          | −                                | +                              | +                                        |
|                              | Saccharomyces cerevisiae  | −                                | +                              | +                                        |
|                              | Kluyveromyces lactis      | −                                | +                              | +                                        |
|                              | Yarrowia lipolecta        | −                                | +                              | +                                        |
| Ciliophora                   | Schizosaccharomyces pombe | −                                | +                              | +                                        |
|                              | Cryptococcus neoformans   | −                                | +                              | +                                        |
| Parasitic Protozoa           | Paramecium tetraurelia    | −                                | +                              | +                                        |
| Kinetoplastida               | Leishmania major          | −                                | +                              | +                                        |
| Apicomplexa                  | Trypanosoma brucei        | −                                | +                              | +                                        |
|                              | Cryptosporidium parvum    | −                                | +                              | +                                        |
|                              | Plasmodium vivax          | −                                | +                              | +                                        |
|                              | Theileria parva           | −                                | +                              | +                                        |
|                              | Trichomonas vaginalis     | −                                | +                              | +                                        |
| Entamoebida                  | Entamoeba histolytica     | −                                | +                              | +                                        |
| Diplomonadida                | Giardia lamblia           | −                                | +                              | +                                        |

*a+, a gene that encodes a possible protein ortholog exists; −, a gene that encodes a possible protein ortholog is not found.*
NAG codons; this may influence overall codon-specific translation efficiency and accuracy (1, 2, 30). The potential translational regulation mediated by the thio-modification of tRNAs may play an important role in cell survival during stressful natural conditions.

Alternatively, the sulfur transfer UBA proteins and the tRNA thio-modification-related Ubi proteins may be responsible for another important function in addition to the thio-modification of cytosolic tRNAs, and this could account for their strong evolutionary conservation. Recent evidence has suggested that Urm1 can also function as a ubiquitin-like protein modifier (31). This modification is called “urmylation” and involves the conjugation of Urm1 to specific target proteins via its C-terminal thiocarboxylate, the formation of which is mediated by Uba4 (25). However, such urmylation has been most frequently observed under oxidative stress conditions that can reasonably induce such conjugation, but it has rarely been observed under other stress conditions (25, 26). Therefore, it remains unclear whether the important role of Urm1 under a wide range of stress conditions is related to urmylation.

The sequence alignments of the tRNA thio-modification-related and the MPT synthesis-related Ubl proteins from various organisms revealed the presence of their characteristic C-terminal six-amino acid motifs, and these motifs are strictly conserved (STLHGG for the former type and PP(I/V/L)SGG for the latter) (supplemental Table 4). Structural analyses of several tRNA thio-modification-related and MPT synthesis-related Ubl proteins (14, 16, 32, 33) have indicated a common “β-grasped” core with protruding C-terminal tails. The short C-terminal tails of these Ubl proteins are flexible (16, 32), but they are functionally important because the C-terminal glycine residues directly participate in Ubl thio-carboxylate reactions. MoeD, which is an E. coli homolog of Cnx7 and is involved in MPT biosynthesis, extends its C-terminal tail toward the active site of MoeB, which is a UBA protein, or MoeE. This was revealed by the crystal structures of the MoeB-MoeD complex (15) and the MoeE-MoeD complex (16). The C-terminal tail of MoeD adopted completely distinct conformations in these two different complexes to fit into its corresponding active sites (16). In the present study, we showed that substitution of the six C-terminal amino acids of AtCnx7 with STLHGG of the tRNA thio-modification-related Ubl proteins resulted in a slight but significant recovery of the thio-modification of cytosolic tRNAs in the urm1Δ yeast mutant. Therefore, the C-terminal tails of the MPT synthesis-related and tRNA thio-modification-related Ubl proteins can be considered key determinants of their specific involvement in the two distinct metabolic pathways. The PP(I/V/L)SGG motif of the MPT synthesis-related Ubl proteins likely fits well into the corresponding active sites of AtCnx6 and HsMOCS2B, which is a eukaryotic MoeE homolog, for MPT synthesis (16). Conversely, the strictly conserved C-terminal STLHGG motif of the tRNA thio-modification-related Ubl proteins may contact an undefined downstream protein(s) for the thio-modification of cytosolic tRNAs. This may explain why many organisms have retained the two distinct types of Ubls for cytosolic tRNA thio-modification and MPT biosynthesis even though both types of proteins can be activated to form a thio-carboxylate at their C-terminus and donate sulfur via the common Cnx5/MOCS3/uba4-type sulfur transfer UBA proteins. As demonstrated previously, two additional conserved proteins, Ncs2 and Ncs6, in S. cerevisiae and the recently found plant Ncs6 ortholog (Rol5) (34) are also required for cytosolic tRNA thio-modification (6); therefore, they are potential candidates for Urm1 downstream sulfur acceptor proteins.

A consensus C(R/K)XG(X/R/D) motif is often found in the active site loop in the ThiF domain-linked rhodanese (35). We determined that the sequence “CRRGNDQ” was commonly included in the sulfur transfer UBA proteins of plants (Cnx5), and “CKLGNDSG” was included in vertebrate proteins of this type (MOCS3) (supplemental Table 5). Although many bacterial MoeB proteins lack this type of RHD domain, AtCnx5 and ScUba4 absolutely require the RHD domain and the MoeB-like domain for their functions in the thio-modification of cytosolic tRNAs (Fig. 2A and supplemental Fig. S2). However, unlike bovine rhodanese, thiosulfate does not seem to be a physiological sulfur donor for ScUba4 because the RHD-like domains of the Cnx5/MOCS3/uba4-type sulfur transfer UBA proteins exhibit 1,000-fold lower thiosulfate sulfurtransferase activity compared with bovine rhodanese (36). Therefore, another factor in addition to thiosulfate may be required to transfer sulfur to a cytosolic Cnx5/MOCS3/uba4-type UBA protein. We showed previously that the sulfur attached to cytosolic tRNAs was derived through the function of mitochondrial Nfs1 (3), but it is still unclear how the sulfur is transported to the cytosol. We are currently attempting to identify any component that mediates sulfur transfer across the mitochondria to the cytosolic sulfur transfer UBA protein.

During the preparation of this manuscript, a report was published demonstrating that in the archaea Haloferax volcanii two Ubl proteins, termed SAMP1 and SAMP2 (37), participate in MoCo biosynthesis and the thio-modification of tRNA, respectively, and that a common UBA-like protein called UbaA can activate both SAMP proteins (20). Therefore, in higher eukaryotic systems, MoCo biosynthesis and the thio-modification of tRNA in the cytosol may have originated at least in part from such an archaeal system. However, there are major differences between the pathways that deliver sulfur to these systems. In eukaryotes, as described above, sulfur must be transported from the mitochondria, which is the most reducing subcellular compartment, to the cytosol, which is a more oxidizing compartment, before it can be utilized by the sulfur transfer UBA proteins. Interestingly, the archaeal UbaA protein does not possess the RHD-like domain sequence that is common among the Cnx5/MOCS3/uba4-type sulfur transfer UBA proteins. Therefore, eukaryotes have likely developed several unique mechanisms to protect sulfur against unfavorable oxidation during mobilization. Most of these mechanisms have not yet been elucidated.

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