A Novel N-Methyltransferase in Arabidopsis Appears to Feed a Conserved Pathway for Nicotinate Detoxification among Land Plants and Is Associated with Lignin Biosynthesis

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The Preiss-Handler pathway, which salvages nicotinate (NA) for NAD synthesis, is an indispensable biochemical pathway in land plants. Various NA conjugations (mainly methylation and glycosylation) have been detected and have long been proposed for NA detoxification in plants. Previously, we demonstrated that NA O-glucosylation functions as a mobilizable storage form for NAD biosynthesis in the Brassicaceae. However, little is known about the functions of other NA conjugations in plants. In this study, we first found that N-methyl nicotinate is a ubiquitous NA conjugation in land plants. Furthermore, we functionally identified a novel methyltransferase (At3g53140; NA MT), which is mainly responsible for N-methyl nicotinate formation, from Arabidopsis (Arabidopsis thaliana). We also established that trigonelline is a detoxification form of endogenous NA in plants. Combined phylogenetic analysis and enzymatic assays revealed that NA N-methylation activity was likely derived from the duplication and subfunctionalization of an ancestral caffeic acid O-methyltransferase (COMT) gene in the course of land plant evolution. COMT enzymes, which function in S-lignin biosynthesis, also have weak NAMT activity. Our data suggest that NA detoxification conferred by NAMT and COMT might have facilitated the retention of the Preiss-Handler pathway in land plants.

NAD is a ubiquitous coenzyme that serves as an electron carrier in hundreds of redox reactions. Multiple redox reactions related to core energy metabolism are NAD dependent, including reactions in glycolysis, the Krebs cycle, and the Calvin cycle. Common redox

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two-reaction pathway in which NAM is converted directly to nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase, whereupon NMN is then converted to NAD by nicotinamide mononucleotide adenylyltransferase. This two-step pathway does not include the production of NA, and no close homologs of the Preiss-Handler pathway genes NIC or NaPRT have been identified in the genomes of green algae or animals (Rongvaux et al., 2003; Noctor et al., 2006; Li et al., 2015b). The overaccumulation of NA has been demonstrated to be toxic to plant cells (Zheng et al., 2005; Wang and Pichersky, 2007; Li et al., 2015b). We previously proposed that plants evolved various strategies to deal with NA toxicity based on the fact that various conjugates of NA (glycosylation or methylation at the N-position or carboxyl group of NA) have been detected in plants. We further demonstrated that NA O-glycosylation functions to detoxify NA and that this process is likely restricted to the Brassicaceae (Li et al., 2015b).

Trigonelline (Tg; N-methylnicotinate), an alkaloid that was named after its isolation from the seeds of Trigonella foenum-graecum, has been found in a wide range of plant species from ferns to flowering plants (Matsui et al., 2007; Ashihara et al., 2012; Zhou et al., 2012; Li et al., 2015b). Tg also is known to be beneficial to human health by affecting Glc metabolism and lowers the risk of type 2 diabetes, and it accounts for approximately 1% of the dry matter in coffee (Coffea arabica) beans (Allred et al., 2009; van Dijk et al., 2009). The potential use of Tg in other therapies was reviewed recently (Zhou et al., 2012). Tg is synthesized from NA, the intermediate metabolite in the Preiss-Handler pathway. However, little is known about its physiological functions in plants. Tg came to the attention of plant biologists because it appeared to function as a plant hormone and was proposed to have a role as a cell cycle regulator in roots and shoot meristems (Evans et al., 1979). Subsequently, multiple studies established that Tg likely functions as a plant growth regulator in diverse processes such as nodulation, abiotic stress responses, DNA methylation, and nyctinasty (Minorsky, 2002). However, such ideas lacked supporting evidence at the genetic and molecular levels because no gene for a Tg synthase had been identified. A nicotinate N-methyltransferase (NANMT) protein was partially purified from heterotrophic cell suspension cultures of soybean (Glycine max) and biochemical studies established that NANMT, like other plant natural product methyltransferases, uses SAM as a methyl donor (Upmeier et al., 1988). However, no NANMT cDNA sequence has been cloned from soybean to date. Recently, two NANMT cDNAs (named CTgS1 and CTgS2, which belong to the SABATH methyltransferase family [also referred to as motif B’ methyltransferase]) were functionally identified in coffee (Mizuno et al., 2014). We previously screened all 24 Arabidopsis (Arabidopsis thaliana) SABATH proteins for catalytic activity with 59 potential substrates, including NA, and no NANMT activity was detected (Yang et al., 2006a, 2006b). One AtSABATH protein encoded by At5g04370 methylated NA at the carboxyl group rather than the N-position to form NA methyl ester (Yang et al., 2006a). These studies suggested that another type of methyltransferase was recruited for Tg production in Arabidopsis, illustrating a case of convergent evolution in plant specialized metabolism (Pichersky and Lewinsohn, 2011).

Since the first structure of a plant natural product methyltransferase (chalcone O-methyltransferase) was reported in 2001, the structures of several plant methyltransferases with diverse methyl acceptor products have been elucidated (Zubieta et al., 2001; Gang et al., 2002; McCarthy and McCarthy, 2007; Louie et al., 2010). These studies revealed that all plant SAM-dependent methyltransferases share a highly conserved structure (especially the SAM-binding domain), although they have little sequence identity (Martin and McMillan, 2002; Liscombe et al., 2012). This structural property of plant methyltransferases and the relatively large size of methyltransferase gene families in plants (e.g. 478 genes

Figure 1. Tg biosynthesis and NAD metabolism in land plants. The step catalyzed by NANMT is indicated in blue. AO, Asp oxidase; NaAD, nicotinate adenine dinucleotide; NaMN, nicotinate mononucleotide; PARP, poly(ADP-ribose) polymerase; QPT, quinolinate phosphoribosyltransferase; QS, quinolinate synthase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SIRT, sirtuins (NAD-dependent protein deacetylases).
were found in a search of the Arabidopsis genome annotation database using the search term methyltransferase (www.arabidopsis.org) make it very difficult to predictively determine the particular substrate(s) of a given methyltransferase. In mammals, the nicotinamide N-methyltransferase (hNNMT; GenBank accession no. U08021) has been characterized biochemically and structurally (Aksoy et al., 1994; Peng et al., 2011). Although the chemical structure of NA is similar to that of NAM (Fig. 1), no hNNMT homologs (greater than 20% identity at the protein level) could be found in any plant genome.

In this study, using gene-enzyme correlation analysis (Li et al., 2015b), we identified a novel methyltransferase (not a SABATH methyltransferase) that is responsible mainly for Tg production in Arabidopsis. We demonstrate that the in planta physiological functions of Tg include the detoxification of endogenous NA and involvement in NAD homeostasis. Phylogenetic analysis and biochemical characterization of related methyltransferase proteins from 10 plant species, selected based on their positions at important evolutionary nodes, clearly indicated that the conserved NANMT proteins probably evolved from plant caffeic acid O-methyltransferases (COMTs), which also have weak NANMT activity across the land plants. Based on our results, we propose that NA detoxification conferred by NANMT and COMT facilitated the retention of the Preiss-Handler pathway in land plants. Our discovery also paves the way for investigations of other physiological functions of Tg in land plants.

RESULTS

Tg Is Detected in Basal Land Plants

Although Tg has been detected in a wide range of vascular plants (Ashihara et al., 2012; Li et al., 2015b), our knowledge of the distribution of Tg in basal land plants is still lacking. Thus, we used two methods to test...
whether Tg is produced in basal land plants and the green alga *C. reinhardtii* strain cc400. We fed different organs of *Physcomitrella patens* (Bryophytes) and *Selaginella moellendorfii* (Pteridophytes) with 10 μM [14C]NAM and assayed for Tg synthase activity in planta (Supplemental Fig. S1). We also used liquid chromatography triple quadrupole mass spectrometry (LC-QQQ-MS) to analyze the endogenous Tg content in *S. moellendorfii* and *P. patens* at both the protonema and sporophyte stages. Although no clear signal for [14C]Tg was detected in the [14C]NAM feeding experiments (Fig. 2A), the LC-QQQ-MS analysis showed that endogenous Tg was clearly present in both *S. moellendorfii* (0.31 ± 0.094 nmol g⁻¹ fresh weight; n = 3) and *P. patens* (0.039 ± 0.0093 nmol g⁻¹ fresh weight in protonema material and 0.047 ± 0.0045 nmol g⁻¹ fresh weight in sporophyte material; n = 3; Fig. 2, B–D). These results suggest that Tg is widely distributed in land plants.

**Functional Characterization of NANMT from Arabidopsis**

We previously profiled NA conjugates in seven different tissues of Arabidopsis and found that [14C]Tg accumulates (reflecting NANMT activity) predominantly in inflorescence tissues but also is present in stems and siliques (see Fig. 1 in Li et al., 2015b). Here, we used the same strategy (gene-enzyme correlation analysis) to identify eight candidate genes (*At4g10440*, *At1g67990*, *At3g53140*, *At5g53810*, *At5g51130*, *At5g37170*, *At3g51070*, and *At1g04050*) from a total of 289 annotated methyltransferase probes (P < 0.001; Supplemental Data Set S1). Open reading frames were finally obtained for six candidate genes, and the protein encoded by *At3g53140* clearly had the N-methyltransferase activity with NA as a substrate (Fig. 3). Therefore, *At3g53140* was designated as AtNANMT1. AtNANMT1 did not show any detectable activity with NAM in the same enzymatic assay. AtNANMT1 displayed similar levels of activity at pH values ranging from 5 to 8, and NANMT activity was not stimulated significantly by the presence of various metal ions (Supplemental Fig. S2). NANMT had an apparent Kₘ value of 38.7 ± 1.99 μM (n = 3) for NA, and its K₅ₐ value was 3.52 ± 0.082 s⁻¹ (n = 3; Table I).

To deepen our understanding of the in planta function of Tg, two types of transgenic plants were generated. First, two independent lines with T-DNA insertions in...
AtNANMT1 were isolated and characterized; no NANMTI transcripts could be detected in either mutant. Crude protein extracts were prepared from inflorescence tissue where AtNANMT1 was highly expressed but did not show any NANMT activity (Supplemental Fig. S3, A–C). Therefore, these two null mutant alleles were designated as nanmt1-1 (SALK_046243) and nanmt1-2 (SALK_071460). Second, transgenic plants (Columbia background [Col-0]) expressing (SALK_071460). Second, transgenic plants (Columbia background [Col-0]) expressing AtNANMT1 under the control of the cauliflower mosaic virus 35S promoter were generated and characterized. Two independent lines (OE-1 and OE-2) with high NANMT activity in crude protein extracts from young seedlings were selected for further experiments (Supplemental Fig. S3, D and E).

Homozygous nanmt1-1 and nanmt1-2 lines showed marked reductions in Tg accumulation in inflorescence tissues, whereas the levels of NA, glucosylated NA (NAGO and NANG), NAMN, and NAD were increased significantly (Fig. 3C). As expected, relative to wild-type plants, the OE-1 and OE-2 lines had higher Tg content and lower NA, NAGO, NANG, NAMN, and NAD contents (Fig. 3C). Although the chemical profiles were altered dramatically, the expression patterns of genes involved in NAD biosynthesis and NA modification were largely unchanged in inflorescence tissues, with the exception of NCI1, whose expression increased by 2-fold in the OE-1 and OE-2 lines (Supplemental Fig. S4). Similar patterns of NA, Tg, NAG, and NANG accumulation also were found in the developing seeds of all tested plants (Supplemental Fig. S5).

Quantitative PCR analysis verified that AtNANMT1 was expressed at the highest level in inflorescence tissue, a finding consistent with the strong accumulation of Tg in inflorescences in the Tg profiling experiments (Fig. 4A). Analysis of plants expressing a ProNANMTI::GUS transgene further confirmed the inflorescence-specific expression pattern of AtNANMT1. AtNANMT1 expression was especially strong in anthers, developing siliques, and developing seeds (Fig. 4B). The subcellular localization of the NANMT1::GFP fusion protein in protoplasts indicated that AtNANMT1 is a cytosolic protein (Fig. 4C).

Taken together, the results of these functional characterization experiments enable us to conclude that NANMT1 encoded by At3g53140 is the enzyme responsible for Tg biosynthesis in Arabidopsis. It should be noted that low but detectable levels of Tg were found in nanmt1 mutants (inflorescence tissue and developing seeds; Fig. 3C; Supplemental Fig. S5), suggesting that the loss of AtNANMT1 activity does not completely block the production of Tg in Arabidopsis.

**NANMT Is Involved in NA Detoxification in Arabidopsis**

None of the AtNANMT1 transgenic plants showed obvious phenotypic abnormalities, even in the inflorescence tissue where NANMT1 was most highly expressed (Supplemental Fig. S6). However, we did find that seedlings of the OE-1 (6.75 ± 0.4 cm in root length; n = 4) and OE-2 (7.3 ± 0.68 cm; n = 4) lines grew much faster (as measured by root length) than wild-type (5.38 ± 0.6 cm; n = 4) or nanmt1 (5.63 ± 0.69 cm for nanmt1-1 and 5.22 ± 0.52 cm for nanmt1-2; n = 4) plants when grown on one-half-strength Murashige and Skoog (1/2 MS) medium plates supplemented with 100 μM NA (Fig. 5, B and D). In contrast, Tg treatment did not inhibit the root growth of any of the tested lines, and no significant differences in

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**Table 1. Catalytic efficiency of plant NANMT enzymes**

All data obtained in this study are presented as means ± s.d from triplicate independent assays. N/A, Not applicable.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
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</thead>
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<tr>
<td>AtNANMT1</td>
<td>NA</td>
<td>38.70 ± 1.99</td>
<td>3.52 ± 0.82</td>
<td>0.91 ± 0.0063</td>
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<td></td>
<td>SAM</td>
<td>52.13 ± 2.51</td>
<td>3.01 ± 0.60</td>
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<td>Gm13G263200</td>
<td>NA</td>
<td>55.86 ± 3.08</td>
<td>4.81 ± 0.86</td>
<td>0.086 ± 0.0038</td>
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<tr>
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<td>SAM</td>
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<td>4.14 ± 0.91</td>
<td>0.060 ± 0.0027</td>
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<td>Os02g57760</td>
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<td>43.62 ± 0.91</td>
<td>2.25 ± 0.12</td>
<td>0.051 ± 0.0010</td>
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<tr>
<td></td>
<td>SAM</td>
<td>67.26 ± 4.06</td>
<td>2.07 ± 0.19</td>
<td>0.031 ± 0.0015</td>
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<td>Aco_009_01073</td>
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<td>5.83 ± 0.11</td>
<td>0.086 ± 0.0078</td>
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<tr>
<td></td>
<td>SAM</td>
<td>69.83 ± 5.64</td>
<td>4.44 ± 0.14</td>
<td>0.064 ± 0.0032</td>
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<td>PineTC169872</td>
<td>NA</td>
<td>60.58 ± 1.66</td>
<td>4.97 ± 0.71</td>
<td>0.082 ± 0.0021</td>
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<td></td>
<td>SAM</td>
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<td>Sly10g085830</td>
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<td>SAM</td>
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<td>Tca1EG0223411</td>
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<td>SAM</td>
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<td>Crude protein</td>
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<td></td>
<td>SAM</td>
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*Kinetic parameters for NA were determined with 500 μM SAM. **Kinetic parameters for SAM were determined with 200 μM NA. †The crude protein was prepared from inflorescences of 6-week-old Arabidopsis plants.
Previously, Shimizu and Mazzaferra (2000) found that Tg was demethylated for NAD biosynthesis during the early stages of coffee seed germination. To determine the metabolic fate of Tg in Arabidopsis, we fed four Arabidopsis tissues (2-week-old seedlings, cauline leaves, inflorescences, and siliques) with purified [carboxyl-14C]Tg for 20 h. However, only [14C]Tg, and no [14C]NAD or other labeled chemicals, was detected in this experiment (Supplemental Fig. S7). This result indicates that Tg cannot be reutilized for NAD biosynthesis, which is consistent with the results of labeling experiments in mung bean (Vigna radiata; Zheng et al., 2005).

**Phylogenetic and Enzymatic Analyses of Plant NANMTs**

To trace the evolutionary history of plant NANMTs, we constructed a phylogenetic tree of NANMT1 homologs and related methyltransferase proteins from all available plant genomes (Fig. 6A). Close homologs of AtNANMT1 could be found in all seed plants (greater than 40% protein identity to AtNANMT1). The closest homolog of AtNANMT1 in Arabidopsis is AtCOMT1 (encoded by At5g54160; 38% identity to AtNANMT1); this protein has been demonstrated to be responsible for 5-lignin formation in planta (Goujon et al., 2003). The NANMT-like proteins and COMT-like proteins from seed plants are clearly separated. It is noteworthy that the related OMT proteins from the basal land plants (nine OMT proteins from S. moellendorfii and three OMT proteins from P. patens) have similar protein sequence identity (30–40%) to both NANMT-like proteins and COMT-like proteins from seed plants. No close homologs of NANMT and COMT could be found in C. reinhardtii (less than 20% protein identity). Despite the fact that there are high levels of Tg accumulation in coffee beans, no AtNANMT1 homolog could be found in the recently published coffee genome (Coffee canephora; http://coffee-genome.org/ [Denoeud et al., 2014]). It should be noted that NANMT is a single-copy gene in the sequenced genomes analyzed in this study, except for in soybean, where two copies of the NANMT gene were found (Fig. 6A).

We next conducted enzymatic assays with NANMT-like proteins from 10 representative plant species, selected based on the phylogenetic tree in Figure 6A (Matsui et al., 2007; Ashihara et al., 2012). All of the NANMT-like proteins from seed plants had high catalytic efficiency toward NA, with the exception of the NANMT-like protein from Amborella trichopoda, a basal angiosperm plant (Fig. 6B; Table I). Interestingly, AtCOMT also showed weak NA N-methylation activity (~1.6% of COMT activity toward caffealdehyde; Fig. 6B; Table II). Among the 13 OMTs from basal land plants that we assayed, only Ppa12g019400, Smo438615, and Smo227179 showed NA N-methylation activity, and this activity was extremely weak (Fig. 6C; Table II). Ppa12g019400 and Smo438615, like AtCOMT, showed much higher phenylpropanoid methylation activity than NA N-methylation activity (Table II; Supplemental Fig. S8). However, Smo227179 showed specificity for NA; it showed no activity toward phenylpropanoid substrates (Table II).
Multiple sequence alignment revealed that NA\textsubscript{MT}s contain all of the signature motifs that are conserved among OMT enzymes (Supplemental Fig. S9). To further explore structure-function relationships among the various NA\textsubscript{MT} proteins, an AtNA\textsubscript{MT1} structural model was generated based on the published crystal structures of COMTs from perennial ryegrass (\textit{Lolium perenne}) and alfalfa (\textit{Medicago sativa}; Zubieta et al., 2002; Louie et al., 2010). Structural analysis suggested that there is one catalytic residue, Thr-264, and three substrate-binding residues, Asn-21, Tyr-120, and His-124, in AtNA\textsubscript{MT1} (Fig. 7A). The structure of the AtNA\textsubscript{MT1} protein docked with SAH and NA also suggested that the carboxyl group (negatively charged)}

**Figure 5.** Root growth and chemical analysis of Col-0, \textit{nanmt1} mutants, and AtNA\textsubscript{MT1} OE lines exposed to NA and Tg. A to C, Four-day-old seedlings, with 10-mm roots, of Col-0, \textit{nanmt1} mutants, and AtNA\textsubscript{MT1} OE lines were transferred from 1/2 MS plates to plates supplemented with 100 \textmu M NA or Tg and grown for 7 additional days. D, Root growth of seedlings grown under 100 \textmu M NA or Tg treatment. Data are presented as means ± SD of four independent experiments (three seedlings of each line were used in one experiment). Asterisks indicate significant differences from Col-0 plants in the same treatment: **, \textit{P} < 0.01 (two-tailed Student’s \textit{t} test). E, Chemical analysis of the seedlings of AtNA\textsubscript{MT1} transgenic plants under various growth conditions (1/2 MS, NA treatment, and Tg treatment). Bars show means ± SD (\textit{n} = 4). Asterisks indicate significant differences from wild-type plants: *, \textit{P} < 0.05 and **, \textit{P} < 0.01 (two-tailed Student’s \textit{t} test). F.W., Fresh weight.

**Homology Modeling of NANMT**

Multiple sequence alignment revealed that NANMTs contain all of the signature motifs that are conserved among OMT enzymes (Supplemental Fig. S9). To further explore structure-function relationships among the various NANMT proteins, an AtNANMT1 structural model was generated based on the published crystal structures of COMTs from perennial ryegrass (\textit{Lolium perenne}) and alfalfa (\textit{Medicago sativa}; Zubieta et al., 2002; Louie et al., 2010). Structural analysis suggested that there is one catalytic residue, Thr-264, and three substrate-binding residues, Asn-21, Tyr-120, and His-124, in AtNANMT1 (Fig. 7A). The structure of the AtNANMT1 protein docked with SAH and NA also suggested that the carboxyl group (negatively charged)
Figure 6. Phylogenetic analysis and biochemical characterization of NAMT-like proteins from diverse land plant taxa. A, Phylogenetic tree of AtNAMT1 and AtCOMT1 (encoded by At5g54160) and their homologs from different plant lineages. The
of NA forms a single hydrogen bond interaction with the imidazole group of the side chain of His-124 (positively charged), which is critical for the substrate specificity of AtNANMT1 toward NA. The replacement of the carboxyl group in NA with an amide group in NAM disrupts this hydrogen bond, resulting in the inactivity of NAMTs toward NAM (Supplemental Fig. S10).

To test whether these predicted residues play a critical role in the AtNANMT-catalyzed N-methylation of NA, we changed the Asn-21, Tyr-120, His-124, and Thr-128 to Ser, Leu, His, and Thr, respectively (Fig. 7B). None of the mutated AtNANMT1 proteins had COMT activity. All mutated AtCOMT proteins (S25N, L125Y, N129H, and H267T) had no NANMT activity (Fig. 7C).

### DISCUSSION

We previously demonstrated that free NA, which is toxic to plant cells, emerged in plant metabolism when the Preiss-Handler NAD salvage pathway was evolved in land plants (Li et al., 2015b). The advantage for land plants of using the Preiss-Handler pathway instead of a two-step NAD salvage pathway remains unclear. Plants have evolved strategies to adapt to the toxicity of NA, which represents an endogenous constraint on plant

![Table II. Relative activities (%) of plant NANMT and COMT proteins toward NA and phenylpropanoids](table2.png)

![Figure 6. (Continued)](figure6.png)
growth and development. Consistent with this concept, the capacity to make at least one chemical modification to NA (either glycosylation or methylation of the N-position or carboxyl group of NA) is present in all tested land plants (Matsui et al., 2007; Ashihara et al., 2012; Li et al., 2015b), including those evaluated in this study. NA glycosylation was demonstrated at the genetic level to function in the detoxification of endogenous NA stress in Brassicaceae plants (Li et al., 2015b). Moreover, in this study, we found that Tg biosynthesis represents a conserved detoxification mechanism for managing free NA, an idea supported by the comparison of root growth of AtNANMT1 transgenic plants under NA/NAM treatments (Fig. 5). In addition to our findings relating to the detoxification of NA, our results also show that there is an obvious difference in the relationship of NA glucosylation and NA N-methylation to NAD homeostasis in Arabidopsis; NA N-methylation is clearly involved in the regulation of NAD homeostasis, at least in inflorescence tissue, based on the observation that NAD levels are increased significantly in the nanmt1 mutants and decreased in the AtNANMT1 OE lines (Fig. 3). In contrast, NA glucosylation does not influence NAD homeostasis. This phenomenon could be explained by the fact that AtNANMT1 shows much higher efficiency toward NA and SAM than AtNAGTs show toward NA and UDP-Glc (Li et al., 2015b).

Based on our phylogenetic analysis and biochemical characterization of NANMTs and COMTs from various plant species that are positioned at important evolutionary nodes, we hypothesize that NANMT, which has N-methylation activity, was derived from the duplication of a COMT gene in an ancestral Pteridophytes species. The widespread occurrence of functional COMTs in land plants (from P. patens to flowering plants) observed in our study and the discovery of lignin-like compounds in green algae and red algae also support this hypothesis (Delwiche et al., 1989; Martone et al., 2009; Weng et al., 2011). Additionally, all of the COMTs that we tested had weak NA N-methylation activity (Table II; Supplemental Fig. S8). Arabidopsis COMT1 is known to be a multifunctional enzyme with activity toward both flavonoids and phenylpropanoids (Nakatsubo et al., 2008; Weng et al., 2011). Recently, the capacity to catalyze the methylation of N-acetylsertotonin to form melatonin also was assigned to the Arabidopsis COMT1 protein (Byeon et al., 2014). These observations are consistent with the theory that catalytic promiscuity serves as the starting point for the acquisition of new enzymatic functions; a newly emerged NANMT enzyme likely adopted one of the minor activities of the ancestral COMT (Aharoni et al., 2005; Weng, 2014). Based on our data, we can deduce the phylogeny of NANMT enzymes; the minor NA N-methylation activity was acquired in a COMT duplicated copy in the Bryophytes (Ppa012g019400 in P. patens) after the basal land plant recruited the Preiss-Handler NAD salvage pathway; then, a COMT copy evolved into an NA-specific protein in Pteridophytes (Smo2277279 in S. moellendorffii). Then, the primitive NANMT was evolved into a high-efficiency NA N-methylation enzyme and was retained in higher plants. During this process, several amino acids (such as His-124 and Thr-264 in the AtNANMT1 protein) were likely under positive selection. The weak NANMT activity of the AtCOMT1 protein and the high expression level of AtCOMT1 are likely responsible for the low but detectable levels of Tg in nanmt1 mutant plants (Supplemental Fig. S11). In addition to the NANMT1 identified in this study, there are 12 additional COMT-like proteins in the Arabidopsis genome; these promise to be useful resources for the discovery of other novel methyltransferase activities (Weng et al., 2011). Given the conservation of NANMT in land plants, it is surprising that there appear to be no AtNANMT1 homologs in the coffee genome, as Tg accounts for more than 1% of the dry matter in coffee beans (Allred et al., 2009). However, two SABATH proteins, which share more than 80% identity with coffee caffeine synthase, were identified as NANMT enzymes in coffee (Mizuno et al., 2014). All three enzymes belong to the SABATH gene family, which is distantly related to the COMT and NANMT gene families. It is reasonable to deduce that the coffee NANMT genes evolved more than once (a case of convergent evolution) from the expansion of the
SABATH gene family (23 members in the coffee genome) and that the AtNAMT1 homolog was lost during the evolution of coffee (Pichersky and Lewinsohn, 2011; Denoeud et al., 2014). However, it is not clear whether the gain of NA N-methylation activity from a SABATH gene predates the loss of the NANMT1 homolog or vice versa, although the former is more likely because of the toxicity of N.A. A comprehensive analysis of related enzymes in coffee and related wild relative species will likely be required to answer this question.

In conclusion, we have identified and biochemically characterized a unique group of NANMTs from land plants. These enzymes are involved in NA detoxification and function to fine-tune NAD homeostasis. The residues that are critical for NA N-methylation activity were determined using OMT structural modeling and mutagenesis studies. Our phylogenetic analysis suggests that the recruitment of NA N-methylation activity might have resulted from the duplication of plant COMT genes before the Pteridophytes diverged from the Bryophytes. Finally, we should emphasize that the NA detoxification conferred by NANMT activity may have facilitated the retention of the Preiss-Handler pathway in land plants.

MATERIALS AND METHODS

Plant Materials and Chemicals

The wild-type (ecotype Col-0) and transgenic Arabidopsis (Arabidopsis thaliana) lines used in this study were grown on soil at 22°C under a 16-h-light/8-h-dark cycle. Chlamydomonas reinhardtii (strain cc-400 cw15 mt+) was obtained from the Chlamydomonas Genetic Center (http://www.chlamycc.org). All chemicals used in this study were purchased from Sigma-Aldrich except for the radiolabeled compounds [carboxyl-14C]NAM and [carboxyl-14C]NA (55 mCi mmol⁻¹), which were purchased from American Radiolabeled Chemicals, and [14C]SAM (48.8 mCi mmol⁻¹), which was purchased from PerkinElmer.

MT Gene-NANMT Activity Correlation Analysis

Pearson’s correlation analysis (two tailed) was performed using SPSS software (version 19.0; IBM Software). Transcript expression data for 289 putative methyltransferase genes was bulk downloaded from ATTEDII (version 8.0; http://atted.jp/). Levels of [14C]Tg accumulation (the indicator of NANMT activity) were determined directly from radio-TLC images using ImageJ 1.38e software (downloaded from the U.S. National Institutes of Health; http://rsb.info.nih.gov/ij/). To simplify the analysis procedure, all transcript and metabolite accumulation data were transformed into relative format, meaning that the value of the largest data point was set as 1 (for details, see Supplemental Data Set S1).

AtNAMT1 Expression, Purification, and Enzyme Assays

To generate N-terminally MBP-tagged NANMT, the ORFs of putative NANMT genes from different species were PCR amplified if the plant materials were available, and the appropriate digestion sites were incorporated into the primers used for PCR cloning. Additionally, 13 putative NAMT genes (Aco_009_01073, Atr_00002.374, PineTC169872, Smo85878, Smo96831, Tca1EG022341t1, Tca1ETG022341t1, Tca1GE022341t1, Tca1EG019072, Tca1EG015139, Tca1EG019072, Tca1EG015139), and that the primer information is available). For detailed primer information, see Supplemental Data Set S3).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. P. patens and S. moellendorffii plants and the tissues analyzed in this study.

Quantitative PCR Analysis and GUS Staining

RNA extraction, reverse transcription reaction, quantitative PCR, and Pro-NAMT1:GUS (the ProNAMT1 is approximately 0.5 kb in length) experiments were performed as described previously (Li et al., 2015a, 2015b).

Subcellular Localization of AtNAMT1

Subcellular localization, including the construction of the AtNAMT1-GFP fusion protein (pJIT163-hGFP vector), preparation of Arabidopsis leaf protoplasts, protoplast transformation, and image collection using a laser scanning confocal microscope, was performed as described previously (Xu et al., 2013). For detailed primer information, see Supplemental Data Set S4.

Liquid Chromatography-Mass Spectrometry Analysis

The endogenous levels of NA, Tg, and the NAD-related chemicals were determined with an ultra-performance liquid chromatography-tandem mass spectrometry analytical platform consisting of an Agilent 1290 Infinity liquid chromatography pump and a 6465 triple quadrupole mass spectrometer (Agilent), following the protocol described in our previous study (Li et al., 2015b).

Phylogenetic Analysis

The sequences of AtNAMT1 and ACOMT1 (encoded by At5g54160) and MT proteins with high identity (greater than 40%) to AtNA1 and AtCOMT1 from other plant species were extracted from TAIR (http://www.arabidopsis.org) and Phytozome 10.3 databases (http://www.phytozome.net; for detailed sequence information, see Supplemental Data Set S3). A maximum likelihood tree was constructed using MEGA6.0 software (Tamura et al., 2013).

Homology Modeling and Docking

The AtNAMT1 sequence was used as a query in a BLAST search against the Protein Data Bank (http://www.rcsb.org/pdb/), and six proteins that share 35% to 60% identity with AtNAMT1 were selected (Protein Data Bank identifiers 1FP1, 1KYW, 3PK3, 3TKY, 4PCV, and 5CVJ). Based on the six crystal structure models, we used the MODELER software to construct the protein models with SAH as a cofactor and NA as the substrate (Sali and Blundell, 1993). After model building, the Gromacs version 5.1.2 (www.gromacs.org) program with the CHARMM26 force field was used for local energy minimization. The AtNAMT1 model was set in the center of a water box solvated with simple point charge (SPC)-type water molecules. Additionally, Na⁺ and Cl⁻ ions were placed randomly in the simulation system to neutralize it and to set the NaCl concentration to 0.1 M. Finally, the overall quality factor of the modeled structure was assessed using the ERRAT and PROCHECK programs (Colevo and Yeates, 1993; Laskowski et al., 1993). For protein-ligand docking, simulations were performed using the AutoDockTools and AutoDock Vina, with the protein as a receptor and Tg as a ligand (Morris et al., 2009; Trotter and Olson, 2010), and the top hit was chosen for subsequent biochemical validation. PyMOL (version 1.3) was used for protein model visualization.
Supplemental Figure S2. Assays evaluating the influence of pH and metal ions on the relative activity of AtNA\textsubscript{MT1} (n = 3).

Supplemental Figure S3. Characterization of AtNA\textsubscript{MT1} transgenic plants.

Supplemental Figure S4. Transcript analysis of NAD-related genes in N\textsubscript{ANAMT1} transgenic plants.

Supplemental Figure S5. Chemical characterization of developing seeds (from 9- to 10-week-old silicles) of At\textsubscript{ANAMT1} transgenic plants.

Supplemental Figure S6. Phenotypic analyses of At\textsubscript{ANAMT1} transgenic plants.

Supplemental Figure S7. Metabolic fate of [carboxyl-\textsuperscript{14}C]\textsubscript{J}Tg in different Arabidopsis tissues.

Supplemental Figure S8. Substrate specificity of At\textsubscript{ANAMT}, At\textsubscript{COMT}, Smo\textsubscript{438615}, and Ppa\textsubscript{012g019400} determined using [\textsuperscript{14}C]SAM (40 \mu M) and cold NA or phenylpropanoids (100 \mu M) as cosubstrates.

Supplemental Figure S9. Multiple sequence alignment of N\textsubscript{ANAMT} and COMT\textsubscript{S} from various plant lineages.

Supplemental Figure S10. Radio-TLC analysis of N-methylnicotinamide generated by the N\textsubscript{ANAMT} candidate proteins from representative seed plants using [\textsuperscript{14}C]SAM and SAM as substrates.

Supplemental Figure S11. Tissue specificity of At\textsubscript{COMT} and At\textsubscript{ANAMT1} expression.

Supplemental Data Set S1. Pearson correlation analysis of N\textsubscript{ANAMT} activity ([\textsuperscript{14}C]Tg signal) and expression levels of Arabidopsis methyltransferase genes.

Supplemental Data Set S2. Synthesized genes used in this study.

Supplemental Data Set S3. COMT and N\textsubscript{ANAMT} sequences included in phylogenetic analysis.

Supplemental Data Set S4. Primers used in this study.

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