Auxin methylation by IAMT1, duplicated in the legume lineage, promotes root nodule development in *Lotus japonicus*

Takashi Gotoa,b, Takashi Soyanoa,b, Meng Liua, Tomoko Morig, and Masayoshi Kawaguchia,b,1

*National Institute for Basic Biology, Okazaki, Aichi 444-8585, Japan; and School of Life Science, SOKENDAI (The Graduate University for Advanced Studies), Okazaki, Aichi 444-8585, Japan*

Edited by Eva Kondorosi, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary; received September 16, 2021; accepted January 26, 2022

Legumes attract symbiotic bacteria and create de novo root organs called nodules. Nodule development consists of bacterial infection of root epidermis and subsequent primordium formation in root cortex, steps that need to be spatiotemporally coordinated. The *Lotus japonicus* mutant *daphne* has uncoupled symbiotic events in epidermis and cortex, in that it promotes excessive bacterial infection in epidermis but does not produce nodule primordia in cortex. Therefore, *daphne* should be useful for exploring unknown signals that coordinate these events across tissues. Here, we conducted time-course RNA sequencing using *daphne* after rhizobial infection. We noticed that *IAA carboxyl methyltransferase 1* (*IAMT1*), which encodes the enzyme that converts auxin (IAA) into its methyl ester (MelAA), is transiently induced in wild-type roots at early stages of infection but shows different expression dynamics in *daphne*. *IAMT1* serves an important function in shoot development of *Arabidopsis*, a nonsymbiotic plant, but the function of *IAMT1* in roots has not been reported. Phylogenetic tree analysis suggests a gene duplication of *IAMT1* in the legume lineage, and we found that one of the two IAMT1s (named *IAMT1a*) was induced in roots by epidermal infection. *IAMT1a* knockdown inhibited nodule development in cortex; however, it had no effect on epidermal infection. The amount of root MelAA increased with rhizobial infection. Application of MelAA, but not IAA, significantly induced expression of the symbiotic gene *NIN* in the absence of rhizobial infection. Our results provide evidence for the role of auxin methylation in an early stage of root nodule development.

Legumes develop de novo organs known as root nodules to accommodate symbiotic bacteria called rhizobia. Nodule formation involves two distinct processes: rhizobial infection involving host–microbe communication via signaling molecules in root epidermis, and nodule primordium development accompanied by cell division in root cortex. In epidermis, rhizobia-derived lipochitn oligosaccharide (Nod factor) binds to LysM receptor-like kinases (NFR1/5) in host root hair cells (1–3). This triggers periodic calcium spiking, which is decoded by the calcium/calmodulin-dependent protein kinase (CCaMK) (4–7). CYCLOPS, a direct phosphorylation substrate of CCaMK, acts as a transcription activator of *NODULE INCEPTION* (*NIN*) and *ERF REQUIRED FOR NODULATION* (*ERN*) (8–10), which are necessary to form microcolonies in infection chambers and infection threads (ITs; plant-derived intracellular tube-like structures) (11–15).

Cortical cell division, which occurs just below the site of rhizobial infection in epidermis, is required for primordium development. Phytohormones are important for cortical cell division. Exogenous cytokinin application induces ectopic cortical cell division (16, 17). Some cytokinin receptor genes, such as *Lotus histidine kinase* (*LHK*), are induced in dividing cortical cells upon rhizobial infection (18). Gain-of-function *LHK1* causes spontaneous nodulation (19). Loss of function or knockdown (KD) of *LHK1* or the homologous gene, *Medicago truncatula* CRE1, inhibits nodulation (16, 20). Cortical cell division also provides an indispensable scaffold for IT progression from epidermis to cortex (21), and rhizobia are released from intracellular ITs ramified in nodule primordia, leading to successful nodule organogenesis. Recently, it has been reported that symbiotic communication by callose turnover at plasmodesmata is important for coordinating epidermal infection and nodule development (22). These findings suggest that spatiotemporal coordination across epidermis and cortex is essential for this symbiotic organogenesis. Epidermal expression of genes required for calcium spiking, such as CASTOR and POLLUX (DOES NOT MAKE INFECTIONS 1 [DMI1] in *Medicago*) (23), 24, NUP85 (25), and NUP133 (26), is sufficient for nodule formation (27), suggesting that some kinds of signals generated in epidermis trigger cortical cell division. However, little is known about the mechanism that coordinates these two events.

Among various symbiotic mutants of *Lotus japonicus*, *daphne* is an intriguing nonnodulation mutant in which epidermal infection is uncoupled from cortical cell division (28). In *daphne*, excess ITs are formed in the epidermis but cortical cell division is not activated. The *daphne* mutation is a chromosome translocation 7 kb upstream of the *NIN* start codon, resulting in the ability to promote nodule development (29). This study was supported by a Grant-in-Aid for Young Scientists (A) (17K19829) and a Grant-in-Aid for Scientific Research on Innovative Areas (16H06458) from the Japan Society for the Promotion of Science.

**Author contributions:** T.G., T.S., and M.K. designed research; T.G., M.L., and T.M. performed research; T.G., M.L., and T.M. analyzed data; and T.G. and M.K. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

1To whom correspondence may be addressed. Email: tgtot@nibb.ac.jp or masayoshi@nibb.ac.jp.

This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2116549119/-/DCSupplemental.

Published March 2, 2022.

**Significance**

IAA carboxyl methyltransferase 1 (*IAMT1*) converts auxin (IAA) into its methyl ester (MelAA). *IAMT1* is reportedly critical for shoot development of the nonsymbiotic plant *Arabidopsis*. On the other hand, the function of *IAMT1* in roots is unknown. Here, we found that *IAMT1* is duplicated in the legume lineage, which evolved root nodule symbiosis. In the model legume *Lotus japonicus*, one of two paralogs (named *IAMT1a*) was mainly expressed in root epidermis, but its function is required in the adjacent cell layer, root cortex, where it promotes nodule development. Application of MelAA, but not IAA, significantly induced *NIN*, a master regulator of nodule development, without rhizobia. These findings illuminate our understanding of intertissue communication acquired during evolution of root nodule symbiosis.
a lack of \( NIN \) expression in cortex but not in epidermis. \( NIN \) expression is involved in both epidermal IT formation and initiation of cortical cell division (10, 11, 29), and the regulatory nucleotide sequences for \( NIN \) expression differ between root tissues (28, 30). CYC box, the CYCLOPS binding site, in the \( NIN \) promoter is sufficient for IT formation. The cytokinin response element in the \( NIN \) promoter is required for nodule formation in \textit{Medicago} (30). Cortical \( NIN \) not only induces cortical cell division but also represses excessive infection in epidermis, and a lack of cortical \( NIN \) expression causes the \textit{daphne} phenotype (28). Therefore, in \textit{daphne}, signals derived from infection in epidermis should be overproduced, and signals after cortical \( NIN \)-derived cell division should be reduced. On the other hand, signals that are not reflected in the \textit{daphne} phenotype, including those that induce cortical cell division upstream or independent of cortical \( NIN \) and are derived from epidermal infection, may be overproduced in \textit{daphne}. Therefore, use of \textit{daphne} to explore transcriptional profiles may allow us to uncover genes and factors that coordinate these two events, in addition to the molecular mechanisms of epidermal infection and cortical cell division.

Here, we conducted a time-course transcriptome analysis of \textit{daphne}, and identified genes that showed different expression patterns in \textit{daphne} and wild type (WT). Among these genes, we found \textit{IAA CARBOXYL METHYLTRANSFERASE 1 (IAMT1)}, which encodes the enzyme that specifically converts auxin (indole-3-acetic acid; IAA) into methyl-IAA (MeIAA) (31–33). \textit{IAMT1} is essential for shoot development and differential growth in \textit{Arabidopsis}, a nonsymbiotic plant (34, 35), but, as far as we know, there have been no reports on detailed expression and function analysis of \textit{IAMT1} in roots. In this study, we found that \textit{IAMT1} is duplicated in the legume lineage, and one of the duplicates (named \textit{IAMT1}a) is mainly expressed in epidermis, whereas reverse genetic analysis showed that \textit{IAMT1}a is crucial for nodule development, rather than for epidermal infection. A significant MeIAA increase after rhizobial infection was detected by using \textit{daphne} roots. Furthermore, expression of \textit{NIN} in WT roots increased after MeIAA treatment, in contrast to IAA treatment. Based on these findings, herein we discuss how MeIAA properties differ from those of IAA and how MeIAA may be a signaling molecule that links different events in epidermis and cortex.

**Results**

**Time-Course Transcriptome Analysis of \textit{L. japonicus} MG-20 and \textit{daphne}.

We performed time-course RNA sequencing (RNA-seq) on \textit{L. japonicus} WT MG-20 and \textit{daphne} at early time points after rhizobial inoculation. We set four time points (0 d after inoculation [DAI] [noninoculation], and 1, 2, and 3 DAI): At 1 and 2 DAI, root hair deformation and microcolony entrapment were observed. At 3 DAI, ITs were observed in root epidermis and cortical cell division occurred in WT, while no cortical cell division was observed in \textit{daphne}, despite excessive IT formation.

To identify significant differentially expressed genes (DEGs) during the time course, maSigPro (36) was used. Using a false discovery rate <0.05 as a cutoff, 4,871 genes were classified as time-course DEGs (Dataset S1). Hierarchical clustering of time-course DEGs that changed >2-fold (1,076 genes) revealed four subgroups, based upon expression patterns (Fig. 1A): In cluster I (473 genes), transcript levels increased at 1 DAI in WT but increased to a greater extent and more persistently in \textit{daphne} (Fig. 1B). In cluster II (204 genes), transcription was activated at 1 DAI in \textit{daphne} whereas, in WT, transcription was unchanged or attenuated (Fig. 1B). Cluster III included 222 genes that were more highly up-regulated in WT than \textit{daphne} (Fig. 1B). Cluster IV grouped 177 genes that displayed temporal up-regulation in WT but for which expression was not altered in \textit{daphne} (Fig. 1B). For example, genes associated with infection events in epidermis, such as genes involved in IT formation and/or that act from infected epidermis to cortex, may be included in clusters I and III, which show increased expression in WT. In addition, genes involved in excessive IT formation in \textit{daphne} are most likely to be included in cluster I. On

![Fig. 1. Time-course RNA-seq in WT and \textit{daphne}. (A) Classification of DEGs with fold change \( >2 \) (1,181 genes) into four subgroups (I to IV) by hierarchical clustering. (B) Expression modules of genes with significant differences between WT (black lines) and \textit{daphne} (orange lines) during early infection. Each dot in each cluster represents an average value.](https://doi.org/10.1073/pnas.2116549119)
the contrary, genes that positively regulate nodule primordium formation and/or act repressively from cortex to epidermal infection can be included in cluster IV, where no induction of expression occurs in *daphne*.

**Phylogenetic Analysis and Expression of *L. japonicus* IAMT1.**

*Lj2g3v3222870* was one of the most differentially expressed DEGs in cluster I ($P = 3.97 \times 10^{-9}$). A phylogenetic tree showed that *Lj2g3v3222870* is included in the IAMT1 clade of the SABATH family, which comprises a group of small-molecule methyltransferases (Fig. 2B). IAMT1 encodes an enzyme that specifically converts IAA into its methyl ester (31–33) (Fig. 2A). An asterisk indicates the duplication in the Fabaceae. (D) *Lj2g3v3222870* (*LjIAMT1a*), but not *Lj6g3v0819010* (*LjIAMT1b*), was detected as a DEG in time-course RNA-seq analysis. mRNA abundance of WT (gray bars) and *daphne* (black bars) in *LjIAMT1a* and *LjIAMT1b* at 0 (noninoculation), 1, 2, and 3 DAI. Error bars indicate means ± SDs of three biological replicates.

![Figure 2](https://doi.org/10.1073/pnas.2116549119)

**Fig. 2.** Expression patterns of two IAMT1 genes in *L. japonicus* and phylogenetic trees containing these genes. (A) *Arabidopsis* IAMT1 specifically converts IAA into MeIAA in vitro (31–33). (B) Phylogenetic tree of *Arabidopsis* carboxyl methyltransferases in the SABATH family, including OsIAMT1 (37), and *Lj2g3v3222870*. (C) Fabaceae lineage-specific duplication of IAMT1. An asterisk indicates the duplication in the Fabaceae. (D) *Lj2g3v3222870* (*LjIAMT1a*), but not *Lj6g3v0819010* (*LjIAMT1b*), was detected as a DEG in time-course RNA-seq analysis. mRNA abundance of WT (gray bars) and *daphne* (black bars) in *LjIAMT1a* and *LjIAMT1b* at 0 (noninoculation), 1, 2, and 3 DAI. Error bars indicate means ± SDs of three biological replicates.

Although both *LjIAMT1a* and *LjIAMT1b* highly share a conserved sequence containing the amino acid substitution characteristic of IAMT1 (Fig. 2C).

Despite a highly conserved similarity in the legume lineage, the messenger RNA (mRNA) abundance of *IAMT1a* in roots estimated from RNA-seq data was ~100 times higher than that of *IAMT1b* (Fig. 2D).
**IAMT1a Expression Pattern in the Early Infection Stage.** To determine the genetic dependency of transcriptional changes in IAMT1a, we conducted time-course qRT-PCR experiments on a series of symbiotic mutants after rhizobial infection. In a nin-null mutant (nin-9), as well as in daphne, IAMT1a was induced more highly and continuously than in WT (Fig. 3). This indicates that NIN is at least unnecessary for induction of IAMT1a expression. In contrast, IAMT1a was not induced in ccamk-14 or ern1-6 (Fig. 3), indicating that IAMT1a is induced downstream of CCaMK and ERN2 in the symbiotic pathway.

To identify the expression site of IAMT1a during early rhizobial infection, we performed a histochemical analysis. β-Glucuronidase (GUS) signals driven by the 2.9-kbp IAMT1a promoter in the WT background were detected in the rhizobia-susceptible region at 2 DAI (Fig. 4 B and H). Expression of proIAMT1a:tripleYFP-nls was observed in root epidermis of the susceptible region at the same time (Fig. 4D). However, the GUS signal was attenuated after epidermal ITs were formed (Fig. 4 C, D, and J). These changes in IAMT1a promoter activity were consistent with transient increases in its mRNA levels in WT as detected by RNA-seq and qRT-PCR (Figs. 2D and 3). In contrast, in the daphne background, the susceptible window remains open (28), and GUS signals were detected in the broader root region after inoculation (Fig. 4 F and G). Interestingly, GUS signals were detected in the region in which epidermal IT formation was observed in daphne (Fig. 4K). These patterns are consistent with persistent increases in its mRNA levels in daphne (Figs. 2D and 3).

**IAMT1a Knockdown Affected Cortical Events, but Not Epidermal infection.** To examine involvement of IAMT1a in nodulation, we performed RNA interference (RNAi) KD analysis of IAMT1a in *L. japonicus*. We prepared three constructs for KD that targeted different sequences (5′ untranslated region or coding sequence). IAMT1a and IAMT1b expression levels were analyzed in roots with real-time RT-PCR, 3 wk after inoculation. In roots transformed with RNAi constructs, IAMT1a transcription levels were reduced to less than half of controls \((10^{-4} < P < 10^{-7})\) (SI Appendix, Fig. S3). Transcription levels of IAMT1b also tended to decrease \((0.2 < P < 0.4)\) (SI Appendix, Fig. S3). On average, IAMT1a-RNAi2 reduced IAMT1a transcripts to 10% of control levels. IAMT1a-RNAi2 was the most effective for decreasing IAMT1a transcripts, but IAMT1a-RNAi2 had the weakest effect on reducing IAMT1b transcripts (SI Appendix, Fig. S3). When the number of nodules was measured 3 wk after inoculation, the number of nodules decreased significantly in hairy roots transformed with IAMT1a-RNAi vectors (Fig. 5). Nodules were not observed in 21 to 33% of plants with hairy roots harboring IAMT1a-RNAi vectors, although nodules formed in all controls (Fig. 5). IAMT1a-RNAi also significantly inhibited formation of nodule primordia at 7 DAI (SI Appendix, Fig. S4A). Interestingly, in IAMT1a-RNAi hairy roots without nodules, ITs wandered in epidermis but did not enter cortex (SI Appendix, Fig. S4B). This is similar to the symbiotic phenotype of *L. japonicus* vag1 and daphne mutants (21, 28).

IAMT1a-RNAi seemed not to affect epidermal infection of rhizobia in WT (SI Appendix, Fig. S4C). To further confirm this, we performed IAMT1a-RNAi using a daphne nonnodulating mutant, which has excessive ITs due to deficient negative feedback by cortical NIN. As a result, excessive ITs of daphne were kept in IAMT1a-RNAi hairy roots 2 wk after inoculation without reduction (SI Appendix, Fig. S4C). These results suggested that IAMT1a contributes more to cortical events than to epidermal infection.

**Fig. 3.** Genetic dependencies of IAMT1a expression in the early infection stage. Time-course qRT-PCR analysis of IAMT1a expression in WT, daphne, nin-9, ccamk-14, and ern1-6 at 0 (noninoculation), 1, 2, and 3 DAI. Data are means of three or more biological replicates and are displayed as values relative to WT at 0 DAI. Error bars indicate means ± SDs (n = 12 plants for each biological replicate). Statistical analysis was performed using ANOVA followed by Tukey’s honest significant difference (HSD) test \((P < 0.05)\) in each genetic background. Different letters indicate significant differences. There were no significant differences (n.s) in ccamk-14 and ern1-6.

**Fig. 4.** Spatiotemporal profile of IAMT1a expression in WT and daphne roots inoculated with or without rhizobia. WT (A–D and H–J) and daphne (E–G and K) roots were transformed with proIAMT1a:GUS or proIAMT1a:tripleYFP-nls (I). GUS activity was observed at 0 DAI (noninoculation; A and E) and 2 DAI (B) and after ITs developed (C and F). The arrowhead indicates active GUS sites in WT. DsRed-labeled M. loti was infected in epidermis (D and G). Magnified images of the susceptible region of WT at 2 DAI (H and I) and the root region where epidermal ITs were observed in WT (J) and daphne (K). Images merged with DsRed fluorescence are shown. (Scale bars, 0.5 mm [A–G] and 100 μm [H–K]).
To assess IAMT1a function in nodule development, we performed IAMT1a-RNAi in the absence of rhizobia, using spontaneous nodule formation (snf) mutants such as constitutively expressing a gain-of-function CCAmk<sup>2608S</sup> (snf1-like) (7, 38) or a gain-of-function LHK1 cytokinin receptor (snf2) (19). IAMT1a-RNAi inhibited spontaneous nodulation in snf1-like (SI Appendix, Fig. S5). This indicated that IAMT1a acts downstream of CCAmk, consistent with the fact that IAMT1a expression was not induced in the ccamk mutant after rhizobial inoculation (Fig. 3). On the other hand, IAMT1a-RNAi did not affect spontaneous bump formation in the snf2 mutant (SI Appendix, Fig. S5). This indicates that the function of IAMT1a is not under control of LHK1-mediated cytokinin signaling in nodule development.

**Overexpression of IAMT1a Promoted Nodulation in the tml-4 Mutant.** To investigate whether IAMT1a positively regulates nodule development, we overexpressed IAMT1a. IAMT1a overexpression had no effect on nodule number in WT (Fig. 6). However, in tml-4 mutants, which produce excessive ITs and nodules due to lack of autoregulation of nodulation (39, 40), an increased number of nodules was observed in overexpressed IAMT1a (Fig. 6). In addition, we confirmed the correlation between expression levels of IAMT1a and nodule number (SI Appendix, Fig. S6). These results show that IAMT1a is a positive regulator of nodule development.

**Involvement of Auxin Methylation in Nodule Development.** To clarify the presence of endogenous MeIAA during nodulation, we tried to detect MeIAA before and after rhizobial infection. Identification of endogenous MeIAA is generally difficult, because the amount of MeIAA is much less than that of IAA (35). Therefore, we used daphne, in which rhizobial infection and accumulation of IAMT1a transcripts were enhanced (Figs. 2D and 3). The use of daphne could facilitate the capture of quantitative change of MeIAA during nodulation. First, we confirmed that overexpression of IAMT1a in hairy roots increased MeIAA levels (SI Appendix, Fig. S7A). Then, we detected the critical MeIAA peak especially in infected roots of daphne at 2 DAI (SI Appendix, Fig. S8). We measured amounts of IAA and MeIAA at 0 DAI (noninoculation) and 2 DAI in WT and daphne. Although no significant change in the amount of IAA or MeIAA could be detected in WT before or after rhizobial infection, a significant MeIAA increase after rhizobial infection was detected in daphne (Fig. 7A). Furthermore, we performed constitutive expression of MES17, which encodes the enzyme that converts MeIAA to IAA (Fig. 7B) (41), to counteract the catalytic function of IAMT1a during nodulation. Constitutive expression of Lj2g3v2171910, a gene homologous to Arabidopsis MES17 (SI Appendix, Fig. S9), resulted in a statistically significant decrease in MeIAA levels and nodule number compared with WT (Fig. 7C and SI Appendix, Fig. S7B). These data indicate the importance of auxin methylation in nodule development. Furthermore, to gain insight into the role of auxin methylation, we tested the effect of exogenous MeIAA on NIN expression. NIN is a key transcription factor of cortical cell division for nodule development (29). Treatment with IAA did not induce NIN expression in L. japonicus roots (Fig. 7D), consistent with findings of Soyano et al. (42). However, treatment with MeIAA did induce NIN expression (Fig. 7D). This induction of expression was not detected in daphne (Fig. 7E), suggesting that MeIAA affects cortical NIN expression. Finally, NIN expression was induced at 7 DAI in hairy roots harboring control vectors, but was poorly induced in hairy roots harboring IAMT1a-RNAi constructs (Fig. 7F). These findings indicate that auxin methylation by IAMT1a is involved in nodule development by affecting NIN expression.

**Fig. 5.** IAMT1a-RNAi inhibits nodulation. (A) Representative phenotype of hairy roots of WT harboring an empty vector (EV) as a control (Left) and the IAMT1-RNAi-2 vector (Right) 3 wk after inoculation. Roots expressing green fluorescent protein as a transformation marker were selected. Root nodules are indicated by arrowheads. (Scale bars, 1 cm.) (B) The nodule number in hairy roots harboring an EV (controls) and IAMT1-RNAi vectors 3 wk after inoculation. Each dot represents the nodule number of each plant. n = 37 (control), 40 (RNAi-1), 40 (RNAi-2), and 28 (RNAi-3). Asterisks indicate that differences are statistically significant (Welch’s t test).
Discussion

IAMT1 has been characterized as a gene encoding carboxy methylestertransferase, which specifically converts IAA to MeIAA in vitro (31–33). In Arabidopsis, a nonsymbiotic plant, IAMT1 participates in MeIAA biosynthesis in vivo (35) and in shoot development and differential growth (34, 35). On the other hand, the function of IAMT1 in roots is unknown. This study demonstrates that L. japonicus IAMT1 functions in root nodule development. We found an IAMT1 gene duplication in the Fabaceae lineage and characterized one of two IAMT1 genes, named IAMT1a, induced in roots after rhizobial infection, as a positive regulator of nodule development. Notably, we identified the increase of MeIAA in roots after rhizobial infection using daphne (Fig. 7A and SI Appendix, Fig. S8). Because MeIAA is much less abundant than IAA (35), a quantitative change of endogenous MeIAA in biological processes has not been reported. In this study, however, the use of daphne allowed us to detect a significant increase of MeIAA levels associated with induction of IAMT1a expression mediated by rhizobial infection.

We documented induction of IAMT1a expression in nodulation using time-course RNA-seq in early symbiotic stages using daphne. IAMT1a is one of the most significant genes in a cluster of DEGs that are transiently induced in WT roots but continuously and more strongly expressed in daphne roots after rhizobial infection (Fig. 1B). Consistent with changes of IAMT1a transcript levels detected using RNA-seq and qRT-PCR, upon epidermal infection, IAMT1a promoter activity is transiently observed in a local infectable region, but not throughout entire roots in WT, whereas it is persistently observed in wide regions of daphne roots (Figs. 2D, 3, and 4). daphne lacks the promoter region of NIN expression in cortex (28), and the ninnull mutant shows persistent expression of IAMT1a, as well as in daphne (Fig. 3), suggesting that characteristic spatiotemporal expression patterns of IAMT1a in daphne result from a lack of cortical NIN. Cortical NIN provides negative feedback and suppresses persistent, widespread epidermal infection (21, 30, 43). Early nodulin 11 is extensively expressed in the Mtn1-1 mutant (10). Given this evidence, IAMT1a expression is probably under negative feedback control by cortical NIN.

A BLAST search of legumes and phylogenetically closely related nonlegumes showed that legumes have two IAMT1 genes and a phylogenetic tree suggested that IAMT1a and IAMT1b genes originated from IAMT1 duplication in the common ancestor of legumes (Fig. 2C). IAMT1b, which shares 90% amino acid sequence identity with IAMT1a, also has a conserved amino acid sequence for the auxin-binding pocket (SI Appendix, Fig. S1). Although it is assumed that both share the same molecular function, mRNA levels of IAMT1a are about 10-fold higher than those of IAMT1b in noninoculated roots. IAMT1a, but not IAMT1b, is induced after rhizobial infection (Fig. 2D and SI Appendix, Figs. S2 and S3) and the difference in expression levels then becomes about 400-fold (Fig. 2D). Recently, the existence of genomic clusters, termed symbiotic islands, has been demonstrated in legume genomes, in which symbiotic genes are colocalized (44). In the L. japonicus MG-20 ecotype, IAMT1a is located on chromosome 2 while IAMT1b is on chromosome 6. Establishment of a new gene locus by gene duplication in IAMT1 may have driven IAMT1a expression in roots, further leading to involvement of auxin methylation in nodule development. Considering that Arabidopsis IAMT1 is required for development and differential growth in shoots and that it functions in leaf development and gravitropic reorientation in hypocotyls (34, 35), IAMT1b may have inherited the function of IAMT1a in shoots of nonlegumes.

Auxin is involved in various processes in nodule symbiosis. During early infection stages, auxin biosynthesis occurs in epidermis, and auxin signaling has been observed in infected epidermis (45). Auxin response factor (ARF) 16a participates in IT formation (46). During the postcortical cell-division stage, LjYUCCA1/MiyUC8 and LjYUCCA11/MiyUC2, encoding auxin biosynthetic enzymes, are expressed (47, 48), and these are downstream factors of SHORT INTERNODES/STYISH (STY), required for node emergence (47). GmYUC2a is induced after rhizobial infection and is involved in nodule formation (49). Posttranscriptional control via miR160 regulates ARF10/16/17 in soybean (50) and Medicago (51) in nodule developmental stages.

Considering that auxin biosynthesis occurs in epidermis during early stages of infection, auxin accumulation in epidermis may provide a substrate for IAMT1a in vivo. As evidence to support this, MeIAA levels showed an increasing trend in WT and were significantly elevated in daphne due to rhizobial infection (Fig. 7A), where IAMT1a is extensively expressed (Fig. 4F). In the beginning, we assumed the involvement of IAMT1a in epicellular stages, such as IT formation. However, unexpectedly, IAMT1a KD had no effect on IT number but inhibited nodule and primordium development involving cortical infection (SI Appendix, Figs. S4 and S5). These findings indicate that IAMT1a is involved in cortical events for nodule development. Consistent with this result, spontaneous nodulation with constitutive expression of CCaMK265D [a gain-of-function type of CCaMK (7, 38)] was inhibited by IAMT1a KD (Fig. 6). Since the ccamk mutant shows no induction of IAMT1a expression after infection (Fig. 3), IAMT1a is positioned as a downstream factor of CCaMK. On the other hand, spontaneous nodulation in the snf2 mutant [a gain-of-function mutant of LHKI (19)] was not significantly inhibited by IAMT1a KD (Fig. 6). Given that LHKI expression in cortex but not epidermis is sufficient to restore bump formation in lhki mutants in the absence of rhizobial infection (52), although LHKI is expressed in epidermis and cortex (18), IAMT1a can act either in parallel with or upstream of cytokinin signaling via LHKI in cortex.

Arabidopsis MES17 has been identified as an MeIAA esterase in vitro (41). Constitutive expression of the homolog in
L. japonicus decreased endogenous MeIAA levels and nodule number (Fig. 7C and SI Appendix, Fig. S7B). Nodule development was inhibited by IAMT1a KD (Fig. 5) and further enhanced by its overexpression in the tml background (Fig. 7 and SI Appendix, Fig. S5). These results show that auxin methylation is an important process during nodule development. It is interesting to note that MeIAA has different properties from those of IAA. Soyano et al. (42) found that expression of NIN is not induced by exogenous IAA in L. japonicus, and the present work confirmed that finding (Fig. 7D). In contrast, exogenous MeIAA does induce NIN expression (Fig. 7D). Expression induction does not occur in daphne (Fig. 7E), which lacks the promoter region for cortical NIN expression, and IAMT1a KD inhibited NIN expression during the nodule developmental stage (Fig. 7F), suggesting that MeIAA contributes to induction of cortical NIN expression for nodule development.

**Fig. 7.** Auxin methylation and NIN expression. (A) Relative amounts of IAA and MeIAA at 0 DAI (noninoculation) and 2 DAI in WT and daphne. Error bars indicate means±SDs of three biological replicates (n=40 plants for each biological replicate). (B) MES17 demethylates MeIAA in vitro (41). (C) Nodule numbers in controls and constitutive expression of LjMES17 3 wk after inoculation. Each dot represents the nodule number of each plant. n = 34 (control) and 36 (ox). (D) Relative expression levels of NIN after treatment with dimethyl sulfoxide (DMSO) as mock, IAA (10⁻⁷ M), or MeIAA (10⁻⁷ M) for 24 h in WT. (E) Relative expression levels of NIN after treatment with DMSO as mock or MeIAA (10⁻⁷ M) for 24 h in WT and daphne. Error bars indicate means±SDs of six biological replicates (n = 10 plants for each biological replicate) (D and E). Asterisks indicate that differences are statistically significant (Welch’s t test) (A and C–E). (F) Relative expression levels of NIN in hairy roots harboring an EV as controls and IAMT1a-RNAi-2 vectors at 0 DAI (noninoculation) or 7 DAI. Error bars indicate means±SDs of three or five biological replicates in control or RNAi, respectively (n > 10 hairy roots for each biological replicate). Statistical analysis was performed using ANOVA followed by Tukey’s HSD test (P<0.05) in each genetic background. Different letters indicate significant differences.
Results of these experiments suggest that auxin methylation is not simply due to alteration of auxin homeostasis, and supp

Plasmid Construction and Transformation. Detailed information is provided in SI Appendix, Materials and Methods.

Microscopy. Bright-field and fluorescence microscopy was performed with a BX50 upright microscope (Olympus) or with an A1R confocal microscope (Nikon). Images were acquired and analyzed using DP Controller (Olympus) and NIS Elements (Nikon).

Histochemical Analysis. Hairy roots of WT and daphne were transformed with the GUS-reporter gene fused to the IAMT1a promoter. Roots were incubated in histochemical GUS staining solution (100 mM NaPO₄ pH 7.0, 0.5 mg mL⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 2 mM K₂Fe(CN)₆, 2 mM K₃Fe(CN)₆, and 0.1% Triton X-100) for <60 min at 37 °C after a 10-min vacuum filtration.

Quantiﬁcation of IAA and MeIAA. Forty roots were harvested in each biologically replicative WT and daphne. For preparation of plant extracts, frozen plant material was digested in 400 μL of 80% MeOH containing 60 pmol [3H]-IAA. After digestion, a 1-mL volume was transferred to a tube, and pulverized using a multitube shacker (Yasu1 Kikai) for 2 min at 1,500 rpm and 4 °C. After centrifugation for 3 min at 13,000 rpm and 4 °C, 300 μL of the supernatant was transferred to another tube. Then, 300 μL of hexane was added. After vortexing and centrifugation at 15,000 rpm for 5 min, the lower layer was collected, dried using a centrifugal evaporator, and dissolved in 20 μL of 80% MeOH after centrifugal evaporation.

Mass spectroscopy analysis was performed using a TripleTOF 5600 mass spectrometer (SCIEX), coupled with a microLC 200 System (SCIEX). Metabolites were separated using a HALO fused C18 column (500-μm internal diameter × 5 cm, 2.7-μm particles) with a gradient elution of mobile phase A (0.5% formic acid/H₂O) and mobile phase B (methanol) (0 min: 5% B; 10 min: 95% B; 13 min: 95% B) at an eluent flow rate of 25 μL/min and room temperature (RT). The mass spectrometer was operated in positive-mode electrospray ionization with multiple-reaction monitoring (MRM). MRM transitions are m/z 190.1 to 130.106 for MeIAA, m/z 176.2 to 130.06 for IAA, and m/z 195.1 to 151.091 for [6H]IAA. Source parameters are curtain gas, 25 psi; spray vol-
age, 5.5 kV; temperature, 550 °C; ion-source gas 1, 25 psi; ion-source gas 2, 35 psi.

Application of MeIAA and IAA. With reference to Yang et al. (41), MeIAA and IAA dissolved in 95% ethanol were diluted 1:1,000 in medium to a final concentration of 10⁻¹⁰ M. Five-day-old seedlings transferred to beakers containing either MeIAA or IAA were incubated at RT in the dark, based on Murray et al. (20). After 24 h, roots were harvested to analyze gene expression.

Data Availability. The RNA-seq data reported in this article have been deposited in the DDBJ Sequence Read Archive (accession no. DRA013121).

All study data are included in the article and/or supporting information.

ACKNOWLEDGMENTS. We thank Dr. Hiromu Kameoka (Tohoku University) for bioinformatics, Ms. Sachiko Tanaka (National Institute for Basic Biology; NIBB) for experimental support, and Dr. Naoki Minamino, Dr. Takashi Ueda, Dr. Kensuke Kawaide, Dr. Nao Okuma, and Dr. Mitsutaka Fukudome (NIBB) for discussions. This work was supported by the Functional Genomics Facility (NIBB Core Research Facilities, Data Integration and Analysis Facility (NIBB), Spectroscopy and Bioimaging Facility (NIBB Core Research Facilities), a Grant-in-Aid for Japan Society for the Promotion of Science Research Fellows (Grant 21J10143), and Grants-in-Aid for Scientific Research (Grants 20H03283 and 19K09599).

1. A. Broughton et al., Legume receptors perceive the rhizobial lipochitooligosaccharide signal molecules by direct binding. Proc. Natl. Acad. Sci. U.S.A. 109, 13859–13864 (2012).
2. E. B. Madsen et al., A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425, 637–643 (2003).
3. S. Radutoiu et al., Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature 425, 585–592 (2003).
4. D. W. Ehrehardt, R. Wais, R. S. Long, Calcium spiking in plant root hairs responding to Rhizobium nodulation signals. Cell 85, 673–681 (1996).
5. J. Levy et al., A putative Ca²⁺ and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. Science 303, 1361–1364 (2004).
6. B. R. Sieberer et al., A nuclear-targeted cameleon demonstrates intranuclear Ca²⁺ spiking in Medicago truncatula root hairs in response to rhizobial nodulation factors. Plant Physiol. 151, 1197–1206 (2009).
7. L. Tirichine et al., Deregelation of a Ca²⁺/calmodulin-dependent kinase leads to spontaneous nodule development. Nature 441, 1153–1156 (2006).
8. S. Singh, K. Katzer, J. Lambert, M. Cerni, M. Parniske, CYCLOPS, a DNA-binding transcriptional activator, orchestrates symbiotic root nodule development. Cell Host Microbe 15, 139–152 (2014).
9. M. R. Cerrì et al., The ERN1 transcription factor gene is a target of the CaMIII/CYCLOPS complex and controls rhizobial infection in Lotus japonicus. New Phytol. 205, 323–337 (2017).
Development in Lotus japonicus cytokinin receptors partially redundantly to mediate nodule formation. Plant Cell 26, 678–694 (2014).

L. Tirichine et al., A gain-of-function mutation in a cytokinin receptor triggers spontaneous root nodule organogenesis. Science 315, 104–107 (2007).

J. D. Murray et al., A cytokinin perception mutant colonized by Rhizobium in the absence of nodule organogenesis. Science 315, 101–104 (2007).

T. Suzuki et al., Endoreduplication-mediated initiation of symbiotic organ development in Lotus japonicus. Development 141, 2441–2445 (2014).

R. Gaudioso-Pedraza et al., Callose-regulated symbiotic communication coordinates symbiotic root nodule development. Curr. Biol. 28, 3562–3577.e6 (2018).

J. M. An et al., Medicago truncatula DM1 required for bacterial and fungal symbiosis in legumes. Science 303, 1364–1367 (2004).

H. Imaizumi-Anraku et al., Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. Nature 433, 527–531 (2005).

K. Saito et al., NUCLEOPORINS is required for calcium spiking, fungal and bacterial symbioses, and seed production in Lotus japonicus. Plant Cell 19, 610–624 (2007).

N. Kanamori et al., A nucleoporin is required for induction of Ca2+ spiking in legume nodule development and essential for rhizobial and fungal symbiosis. Proc. Natl. Acad. Sci. U.S.A. 103, 359–364 (2006).

T. Hayashi et al., Rhizobial infection does not require cortical expression of upstream common symbiosis genes responsible for the induction of Ca2+ spiking. Plant J. 77, 146–159 (2014).

E. Yoro et al., A positive regulator of nodule organogenesis, NODULE INCEPTION, acts as a negative regulator of rhizobial infection in Lotus japonicus. Plant Physiol. 165, 747–758 (2014).

T. Soyano, H. Kouchi, A. Hirota, M. Hayashi. Nodule infection directly targets NF-Y subunit genes to regulate essential processes of root nodule development in Lotus japonicus. PLoS Genet. 9, e1003352 (2013).

J. Liu et al., A remote cis-regulatory region is required for NIN expression in the pericycle to initiate nodule primordium formation in Medicago truncatula. Plant Cell 31, 1283–1301 (2019).

J. C. D’Auria, F. Chen, P. Pichersky, "The SABATH family of MTs in Arabidopsis thaliana and other plant species” in Integrative Phytochemistry: From Ethnobotany to Molecular Ecology. J. T. Romeo, Ed. (Elsevier, 2003), chap. 11, pp. 253–283.

C. Zubieta et al., Structural basis for substrate recognition in the salicylic acid carboxyl methyltransferase family. Plant Cell 15, 1704–1716 (2003).

E. Takubo et al., Role of Arabidopsis INDOLE-3-ACETIC ACID CARBOXYL METHYLTRANSFERASE in auxin metabolism. Biochem. Biophys. Res. Commun. 527, 1033–1038 (2020).

G. Qin et al., An indole-3-acetic acid carboxyl methyltransferase regulates Arabidopsis leaf development. Plant Cell 17, 2693–2704 (2005).

M. Abbas et al., Auxin methylation is required for differential growth in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 115, 6864–6869 (2018).

M. J. Nueda, S. Tarazona, A. Conesa, Next maSigPro: Updating maSigPro Bioconductor package for RNA-seq time series. Bioinformatics 30, 2598–2602 (2014).

N. Zhao et al., Structural, biochemical, and phylogenetic analyses suggest that indole-3-acetic acid methyltransferase is an evolutionarily ancient member of the SABATH family. Plant Physiol. 146, 455–467 (2008).

K. Yano et al., CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc. Natl. Acad. Sci. U.S.A. 105, 20540–20545 (2008).

S. Magori et al., Too much love, a root regulator associated with the long-distance control of nodulation in Lotus japonicus. Mol. Plant Microbe Interact. 22, 259–268 (2009).

M. Takahara et al., Too much love, a novel Kelch repeat-containing F-box protein, functions in the long-distance regulation of the legume-Rhizobium symbiosis. Plant Cell Physiol. 54, 433–447 (2013).

Y. Yang et al., Inactive methyl indole-3-acetic acid ester can be hydrolyzed and activated by several esterases belonging to the AtMEs esterase family of Arabidopsis. Plant Physiol. 147, 1034–1045 (2008).

T. Sojano, Y. Shimoda, M. Kawaguchi, M. Hayashi, A shared gene drives lateral root development and root nodule symbiosis pathways in Lotus. Science 366, 1021–1023 (2019).

E. Yoro, T. Suzuki, M. Kawaguchi, CLE-HAR1 systemic signaling and NIN-mediated local signaling suppress the increased rhizobial infection in the daphne mutant of Lotus japonicus. Mol. Plant Microbe Interact. 33, 320–327 (2020).

Y. Peier et al., The root hair "infectome" of Medicago truncatula uncovers changes in cell cycle genes and reveals a requirement for auxin signaling in rhizobial infection. Plant Cell 26, 4680–4701 (2014).

A. Breakspear et al., Epidermal "infectomes" of Medicago truncatula uncover changes in cell cycle genes and reveal a requirement for auxin signaling in rhizobial infection. Plant Cell 26, 4680–4701 (2014).

A. Shrestha et al., Lotus japonicus nuclear factor YA1, a nodule emergence stage-specific regulator of auxin signaling. New Phytol. 229, 1535–1552 (2021).

K. Schiessl et al., NODULE INCEPTION recruits the lateral root developmental program for symbiotic nodule organogenesis in Medicago truncatula. Curr. Biol. 29, 3657–3668.e5 (2019).

Y. Wang et al., GmYUC2a mediates auxin biosynthesis during root development and nitrogen-fixing nodule development in Medicago truncatula. Funct. Plant Biol. 40, 1208–1220 (2013).

M. Mili et al., Inside out: Root cortex-localized LHK1 cytokinin receptor limits epidermal infection of Lotus japonicus roots by Mesorhizobium loti. New Phytol. 222, 1523–1537 (2019).

X. Tan et al., Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 446, 640–645 (2007).

L. Li et al., The possible action mechanisms of indole-3-acetic acid methyl ester in Arabidopsis. Plant Cell Rep. 27, 575–584 (2008).

M. Kawaguchi, Lotus japonicus Miyakojima MG-20: An early-flowering accession suitable for indoor handling. J. Exp. Bot. 70, 3165–3176 (2019).

N. R. Nizampatnam, S. J. Schreier, S. Damodoran, A. Adhikari, S. Subramanian, MicroRNA160 dictates stage-specific auxin and cytokinin sensitivities and directs soybean nodule development. Plant J. 84, 140–153 (2015).

P. Bustos-Sammarco et al., Overexpression of mRK160 affects root growth and nitrogen fixing nodule number in Medicago truncatula. Funct. Plant Biol. 40, 1208–1220 (2013).

M. Mili et al., Inside out: Root cortex-localized LHK1 cytokinin receptor limits epidermal infection of Lotus japonicus roots by Mesorhizobium loti. New Phytol. 222, 1523–1537 (2019).

X. Tan et al., Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 446, 640–645 (2007).

L. Li et al., The possible action mechanisms of indole-3-acetic acid methyl ester in Arabidopsis. Plant Cell Rep. 27, 575–584 (2008).

M. Kawaguchi, Lotus japonicus Miyakojima MG-20: An early-flowering accession suitable for indoor handling. J. Exp. Bot. 70, 3165–3176 (2019).

T. Suzuki et al., LACK OF SYMBIONT ACCOMMODATION controls intracellular symbiont accommodation in root nodule and arbuscular mycorrhizal symbioses in Lotus japonicus. PLoS Genet. 15, e1007865 (2019).

T. Suzuki et al., Positive and negative regulation of cortical cell division during root nodule development in Lotus japonicus is accompanied by auxin response. Development 139, 3997–4006 (2012).

H. Miyazawa et al., The receptor-like kinase KLAVIER mediates systemic regulation of nodulation and non-symbiotic shoot development in Lotus japonicus. Development 137, 4317–4325 (2010).

W. J. Broughton, M. I. Dilworth, Control of leghaemoglobin synthesis in snake beans. Biochem. J. 125, 1075–1080 (1971).

T. Maekawa et al., GIBBERELLIN Control of root cortical cell division in Lotus japonicus. Plant J. 58, 183–194 (2009).

S. Capella-Gutierrez, J. M. Silla-Martinez, T. Gabaldon, trimAl: A tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics 25, 1972–1973 (2009).

L.-T. Nguyen, H. A. Schmidt, A. Von Haeseler, B. Q. Minh, IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32, 268–274 (2015).

S. Kalyaanamoorthy, B. O. Min, T. K. F. Wong, A. Von Haeseler, L. S. Jermiin, ModelFinder: Fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589 (2017).