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Expression of the 1-Aminocyclopropane-1-Carboxylic Acid Deaminase Gene Requires Symbiotic Nitrogen-Fixing Regulator Gene *nifA2* in *Mesorhizobium loti* MAFF303099

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Many soil bacteria contain 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which degrades ACC, a precursor of the phytohormone ethylene. In order to examine the regulation of the *acdS* gene encoding ACC deaminase in *Mesorhizobium loti* MAFF303099 during symbiosis with the host legume *Lotus japonicus*, we introduced the β-glucuronidase (GUS) gene into *acdS* so that GUS was expressed under control of the *acdS* promoter, and we also generated disruption mutants with mutations in a nitrogen fixation regulator gene, *nifA*. The histochemical GUS assay showed that there was exclusive expression of *acdS* in mature root nodules. Two homologous *nifA* genes, mll5857 and mll5837, were found in the symbiosis island of *M. loti* and were designated *nifA1* and *nifA2*, respectively. Quantitative reverse transcription-PCR demonstrated that *nifA2* disruption resulted in considerably diminished expression of *acdS*, *nifH*, and *nifA1* in bacteroid cells. In contrast, *nifA1* disruption slightly enhanced expression of the *acdS* transcripts and suppressed *nifH* to some extent. These results indicate that the *acdS* gene and other symbiotic genes are positively regulated by the NifA2 protein, but not by the NifA1 protein, in *M. loti*. The mode of gene expression suggests that *M. loti acdS* participates in the establishment and/or maintenance of mature nodules by interfering with the production of ethylene, which induces negative regulation of nodulation.

The formation of nitrogen-fixing root nodules is the result of a series of interactions between (*Brady*)*rhizobium* and its legume host plants (8). The host legumes have several mechanisms for regulating nodule formation (28, 36). The plant hormone ethylene is also known to have inhibitory effects on rhizobial infection and the formation of nodule primordia and to limit nodule number (21, 24, 31). Rhizobia often interfere with ethylene biosynthesis in the host plants by means of rhizobitoxine (25, 37, 38) or 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (19, 33) and reduce host ethylene emission to overcome the negative regulation.

ACC deaminase (EC 4.1.99.4) catalyzes the degradation of an ethylene precursor, ACC, into ammonium and α-ketobutyrate (13). The ACC deaminase structural gene (*acdS*) has been found in many rhizosphere bacteria (10, 13), including fast- and slow-growing rhizobia such as *Rhizobium leguminosarum* bv. viciae 128C53K (19), *Bradyrhizobium japonicum* USDA110 (16), *Mesorhizobium loti* MAFF303099 (15), and *M. loti* R7A (32). In the rhizobacterium *Enterobacter cloacae* UW4, promoter analysis of the *acdS* gene showed that expression of this gene requires both ACC and a leucine-responsive regulatory protein (LRP)-like protein and that anaerobic conditions enhance the expression (10). In *B. japonicum* USDA110 and *R. leguminosarum* bv. viciae 128C53K, the *acdS* genes are also probably regulated by an LRP-like protein and a σ^{70} promoter (16, 19).

In *M. loti* MAFF303099, *acdS* was found in the symbiosis island (15). The enhancing effect of the *acdS* gene on nodulation of *Lotus japonicus* MG-20 Miyakojima roots was demonstrated by using an *M. loti acdS* disruption mutant (33). Furthermore, DNA macroarray analysis showed that a clone containing *acdS* (mlr5932) was upregulated in bacteroid cells. The DNA sequence upstream of *acdS* in *M. loti* includes a putative σ^{54} RNA polymerase sigma factor recognition site and a nitrogen fixation regulator NifA protein binding site known as the upstream activating sequence (4, 33). These lines of evidence suggest that the expression of *acdS* in *M. loti* might be coupled with symbiotic nitrogen fixation. In this study, we examined the expression of *M. loti acdS* during symbiosis using specifically manipulated bacteria; in particular, we focused on the regulation by *nifA*.

MATERIALS AND METHODS

Plant materials and growth conditions. *L. japonicus* MG-20 Miyakojima (17) was grown and inoculated with rhizobia as described previously (22).

Bacterial strains, plasmids, transposon, and growth conditions. The bacterial strains, plasmids, and transposon used in this study are listed in Table 1. *M. loti* MAFF303099 and *Escherichia coli* cells were cultured as described previously (22). When required, the media were supplemented with appropriate antibiotics at the following concentrations: for *M. loti*, 50 mg liter^{-1} phosphomycine, 50 mg liter^{-1} streptomycin, 50 mg liter^{-1} spectinomycin, 20 mg liter^{-1} gentamicin, 10 mg liter^{-1} tetracycline, and 50 mg liter^{-1} kanamycin; and for *E. coli*, 100 mg liter^{-1} ampicillin, 50 mg liter^{-1} streptomycin, 100 mg liter^{-1} spectinomycin, 50 mg liter^{-1} kanamycin, and 15 mg liter^{-1} tetracycline. Phosphomycine was used for counterselection of *M. loti* against *E. coli*.

Construction of *gusA* reporter fused with *acdS* gene. The *gusA* gene was amplified from plasmid pmTn5SS*gusA*20 with the following primers: gusA+StuIAAA-1F (5′-AAAAGGCCTATGTTACGTCCTGTAGAAAC-3′) and gusA+StuIAAA-1801R (5′-AAAAGGCCTTCATTGTTTGCCTCCCTGCT-3′). The PCR product was cloned and inserted into StuI-digested pPD1. The plasmid generated, pPD3, was introduced into *M. loti* MAFF303099 (Fig. 1A). Single-crossover mutants

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TABLE 1. Bacterial strains, plasmids, and transposon used in this study

Strain, plasmid, or transposon	Relevant characteristics[a]	Reference or source
Mesorhizobium loti strains		
MAFF303099	Wild type, ACD$^+$ Fix$^+$ Pm^r	15
ML-GUS	MAFF303099 labeled with *gusA* by mTn*5*SS*gusA20*, Pm^r Sp^r Sm^r	This study
ACD-GUS	MAFF303099 labeled with *gusA* fused with the *acdS* gene under regulation of *acdS* promoter, Pm^r Km^r	This study
nifA1 mutant	*nifA1*::Gm derivative of ML-GUS, Fix$^+$ Pm^r Sp^r Sm^r Gm^r Km^s	This study
nifA2 mutant	Δ*nifA2*::Tc derivative of ML-GUS, Fix$^-$ Pm^r Sp^r Sm^r Tc^r Km^s	This study
Escherichia coli strains		
DH5α	Cloning strain	Toyobo Inc., Tokyo, Japan
S17-1	Strain used for conjugation and gene disruption	30
Plasmids		
pBluescriptII KS(−)	Cloning vector, Ap^r	Takara Shuzo Co., Kusatsu, Japan
pBS-GUS	pBluescriptII KS(−) carrying *gusA* amplified from pKW107, Ap^r	This study
pMS246	Plasmid carrying 1-kb gentamicin resistance cassette, Gm^r Ap^r	1
p34S-Tc	Plasmid carrying 1.5-kb tetracycline resistance cassette, Tc^r Ap^r	3
pmTn*5*SS*gusA*20	Plasmid used for transposon mTn*5*SS*gusA*20 insertion, Ap^r Sp^r	35
pK18mob	Plasmid used for cloning and mating, mob, Km^r	29
pPD1	pK18mob carrying 6.6-kb BamHI fragment containing *acdS*, Km^r	33
pPD3	*gusA* inserted into *acdS* gene StuI site of pPD1, Km^r	This study
C229	*M. loti* ordered cosmid clone no. 229 including mll5837 (*nifA2*), Tc^r	11
C230	*M. loti* ordered cosmid clone no. 230 including mll5857 (*nifA1*), Tc^r	11
C232	*M. loti* ordered cosmid clone no. 232 including mlr5905 (*nifH*), Tc^r	11
pKnifA1	pK18mob carrying 4.8-kb EcoRI fragment containing mll5857 (*nifA1*) from C230, Km^r	This study
pKnifA1::Gm	pK18mob carrying 5.7-kb *nifA1*::Gm, Km^r Gm^r	This study
pKnifA2	pK18mob carrying 6.7-kb SmaI fragment containing mll5837 (*nifA2*) from C229, Km^r	This study
pKnifA2::Tc	pK18mob carrying 7.2-kb Δ*nifA2*::Tc, Km^r Tc^r	This study
pFAJ1702	Plasmid used for complementation, Ap^r Tc^r	6
pFAJnifA2	pFAJ1702 containing 6.7-kb *nifA2* fragment from pKnifA2, Ap^r Tc^r	This study
Transposon		
mTn*5*SS*gusA*20	Minitransposon, *gusA*, Sp^r Sm^r	35

[a] ACD, ACC deaminase; Ap^r, ampicillin resistant; Gm^r, gentamicin resistant; Km^r, kanamycin resistant; Pm^r, phosphomycine resistant; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant; Tc^r, tetracycline resistant.

were selected. One of the mutants was designated ACD-GUS and was inoculated into the host plant.

Construction of *M. loti* with constitutive *gusA* gene. The constitutive *gusA* gene driven by the aminoglycoside phosphotransferase (*aph*) promoter from Tn*5* was introduced into *M. loti* MAFF303099 by using minitransposon mTn*5*SS*gusA*20 (35). A β-glucuronidase (GUS)-labeled strain, designated ML-GUS, was used throughout this study because it had the same growth and nodulation capacities as the wild-type strain.

Histochemical localization of GUS activity. At 5 and 10 days after inoculation (DAI), plant roots inoculated with strains ML-GUS or ACD-GUS were evaluated for GUS activity, as described previously (38). The nodulation events, including infection of the host roots, were observed with a stereomicroscope, as described previously (22).

Construction of *nifA1* and *nifA2* mutants. The *nifA1* mutant was constructed by insertion of the gentamicin resistance gene *aacC1* (Gm cassette) (1) into an NdeI site in *nifA1* (mll5857) (Fig. 1B). The *nifA2* mutant was constructed by deletion and insertion of the tetracycline resistance gene *tet*(C) (Tc cassette) (3) into a SalI site in *nifA2* (mll5837) (Fig. 1C). Double-crossover mutants were selected by screening for resistance and sensitivity to appropriate antibiotics. The validity of the mutants was confirmed by reverse transcription PCR (RT-PCR); the *nifA1*and *nifA2* transcripts from the mutants were not detected using primer sets designed on the basis of the sequence downstream of the inserted antibiotic cassette in the *nifA* homologues.

Complementation of the *nifA2* mutant. A 6.7-kb DNA fragment carrying the *nifA2* gene from pKnifA2 was ligated into pFAJ1702 (6). The resulting plasmid, pFAJnifA2, was introduced into the *nifA2* mutant by conjugation. Restoration of *nifA2* transcription in the *nifA2* mutant was confirmed by RT-PCR.

RNA extraction. Total RNA was extracted from *L. japonicus* roots that included nodule primordia at 5 DAI and from roots with mature nodules at 10 DAI by using TRIzol (QIAGEN, Hilden, Germany). In all cases, genomic DNA was removed by DNase I treatment.

Quantification of transcripts. The transcripts of *gusA*, *acdS* (mlr5932), *nifH* (mlr5905), *nifA1* (mll5857), and *nifA2* (mll5837) in symbiotic cells were determined by real-time RT-PCR. For quantification of the transcripts, the assay was performed with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). The sequences of the *gusA*, *acdS*, *nifH*, *nifA1*, and *nifA2* genes were used to design primers and TaqMan probes specific for these genes. The *nifH* gene was used as the representative gene; this gene is induced by the NifA protein under nitrogen-fixing conditions in many diazotrophic bacteria, including rhizobia (34). The PCR primers and TaqMan probes used are listed in Table 2. Relative levels of gene expression were determined using *gusA* as the reference gene. Three independent experiments were performed.

RESULTS

ACC deaminase gene expression. The GUS activity was monitored during nodulation in *L. japonicus* roots inoculated with the ACD-GUS strain at 5 and 10 DAI. At 5 DAI, no significant GUS activity was detected in either infection threads or nodule primordia (Fig. 2B and D). At 10 DAI, GUS activity was detected in bacteroids in the mature nodules (Fig. 2F) but not in infection threads, nodule primordia, or imma-

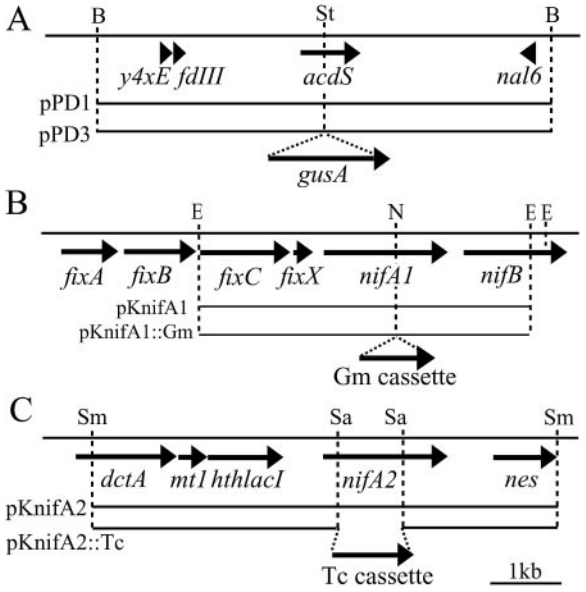

FIG. 1. Physical and restriction enzyme map of the *acdS*, *nifA1*, and *nifA2* genes and the flanking region in *M. loti* MAFF303099. The arrows represent open reading frames. (A) Construction of *gusA* reporter fused with the *acdS* gene. A single-crossover mutation between pPD3 containing an *acdS-gusA* fusion and the genomic DNA generated the ACD-GUS strain harboring an intact genomic *acdS* gene and an *acdS-gusA* fusion. (B) Generation of the *nifA1* mutant. A double-crossover mutation between pKnifA1::Gm and genomic DNA generated the *nifA1* mutant. (C) Generation of the *nifA2* mutant. A double-crossover mutation between pKnifA2::Tc and genomic DNA generated the *nifA2* mutant. B, BamHI; E, EcoRI; N, NdeI; Sa, SalI; Sm, SmaI; St, StuI.

ture white nodules (data not shown). The free-living cells of strain ACD-GUS grown aerobically never exhibited GUS activity. In contrast, the ML-GUS strain in free-living cultures and the infection threads, nodule primordia, and immature and mature nodules of *L. japonicus* inoculated with the same strain consistently exhibited GUS activity (Fig. 2A, C, and E).

Construction of *nifA1* and *nifA2* mutants and their symbiotic phenotypes. The genome of *M. loti* MAFF303099 was found to contain two *nifA* homologue genes, designated *nifA1* and *nifA2*. Therefore, we constructed *nifA1* and *nifA2* mutants

TABLE 2. Primers and probes used for quantitative real-time PCR

Primer or probe	Sequence
Primers	
gusA-1257F	5′-AGGTGCACGGGAATATTTCG-3′
gusA-1317R	5′-ACGCGTCGGGTCGAGTT-3′
nifA1-933F	5′-CGGCCGGTTCGAATTG-3′
nifA1-992R	5′-GAAATCTCGCCGATTTCATCA-3′
nifA2-122F	5′-TCCCTATTGTGCCGTTGCA-3′
nifA2-185R	5′-GCGGTGAGCGCTATCGA-3′
nifH-602F	5′-CCGCCAGACTCAATTCCAA-3′
nifH-662R	5′-GCGTGCTGGACGATGTT-3′
acdS-299F	5′-GCTGGGTTCCACATGAGGAT-3′
acdS-365R	5′-CCCAAGATGCGGCTCAAG-3′
TaqMan probes	
gusA-1279TM	5′-CCACTGGCGGAAGCAACGCG-3′
nifA1-950TM	5′-CGAATGGCGGAACCCTGCTGC-3′
nifA2-143TM	5′-AGAGGGCGCCGGACATCTTTGAAAC-3′
nifH-625TM	5′-ATCCACTTCGTGCCGCGC-3′
acdS-323TM	5′-TCTACGACCGGGTCGGCAACATTC-3′

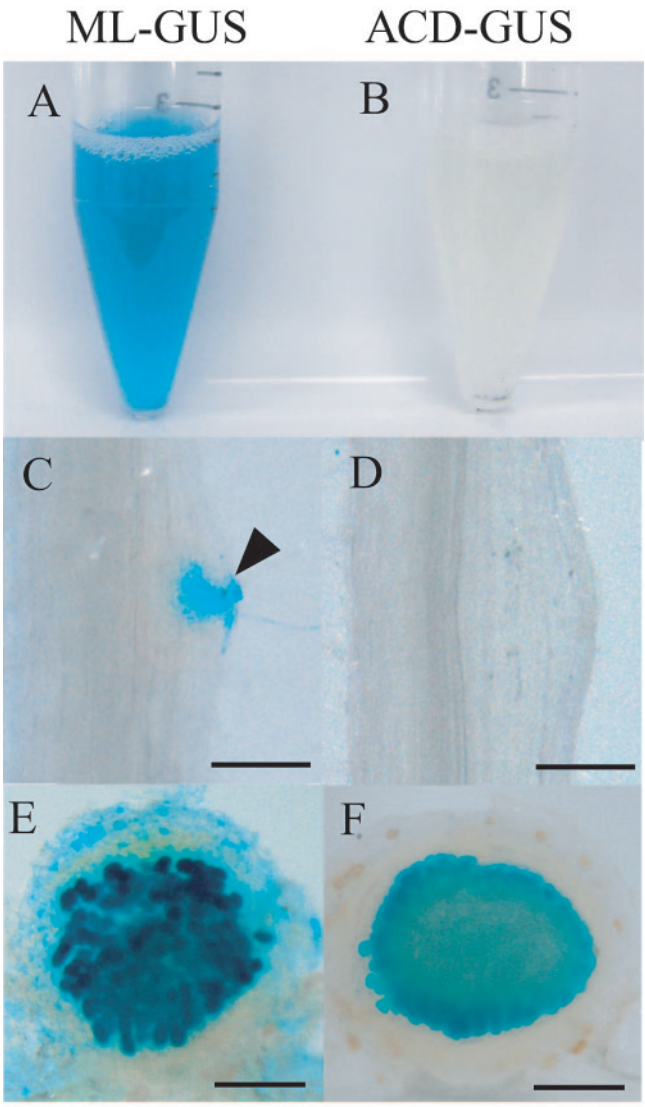

FIG. 2. GUS activity in *M. loti* MAFF303099 expressing *gusA* under control of the constitutively active *aph* promoter (ML-GUS) or the *acdS* promoter (ACD-GUS) in free-living conditions and during nodulation. Constitutively *gusA*-expressing strain ML-GUS (A, C, and E) was used as the positive control for ACD-GUS (B, D, and F). Free-living cell cultures (A and B) and root nodules on *L. japonicus* at 5 DAI (C and D) and 10 DAI (E and F) were incubated with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc). Invasion of cortical cells of nodule primordia by the ML-GUS strain in panel C is indicated by an arrowhead. On the other hand, ACD-GUS showed GUS activity exclusively within mature nodules (F). (C and D) Bars = 100 μm. (E and F) Bars = 300 μm.

(Fig. 1B and C). Plants inoculated with the *nifA1* mutant exhibited a wild-type phenotype (green plants and normal red nodules) (Fig. 3B), like ML-GUS-inoculated plants (Fig. 3A). On the other hand, *nifA2* mutant-inoculated plants had tiny white nodules on the roots and yellow leaves (Fig. 3C). The typical Fix$^-$ phenotype indicated that *nifA2* is essential for symbiotic nitrogen fixation.

Transcription of *acdS*, *nifH*, *nifA1*, and *nifA2* genes during infection. To investigate the expression profiles of the *acdS* and nitrogen fixation (*nif*) genes, the transcript levels of these genes

FIG. 3. Phenotypes of 56-day-old *L. japonicus* plants inoculated with ML-GUS (A), the *nifA1* mutant (B), and the *nifA2* mutant (C) on nitrogen-free medium. Root nodules cut in half are shown in the lower panels. Plants inoculated with the *nifA1* mutant exhibited a wild-type phenotype (B), like ML-GUS-inoculated plants (A). The *nifA2* mutant-inoculated plants had tiny white nodules on the roots and yellow leaves (C). Bars = 600 μm.

in symbiotic cells of *M. loti* MAFF303099 were determined by quantitative RT-PCR. The analysis with the ML-GUS strain showed that transcripts of all four genes had accumulated at 10 DAI (Fig. 4), when mature nodules were present, but not at 5 DAI, when the roots lacked mature nodules (data not shown). The *acdS* expression in mature nodules was consistent with our previous findings (33). The patterns of *nifA* homologues and *nifH* were also consistent with the well-known regulation of *nif* genes dependent on nitrogen fixation.

As shown in Fig. 4, the *nifA2* mutant was not able to transcribe *acdS*, *nifA1*, or *nifH* at 10 DAI. On the other hand, complementation of the *nifA2* mutant with pFAJnifA2 restored *acdS*, *nifH* and *nifA1*expression, indicating that the *nifA2* gene is required for transcription of these genes. In contrast, inoculation with the *nifA1* mutant resulted in a significant increase (2.3-fold) in the *acdS* transcript level and a slight increase (1.3-fold) in the *nifA2* transcript level, whereas there was a significant decrease (0.37-fold) in the *nifH* transcript level (Fig. 4). This result implies that NifA1 may play a secondary role compared with the role of NifA2.

DISCUSSION

Mature nodule-specific and *nifA*-dependent expression of *acdS* in *M. loti*. In this study, we examined the transcriptional regulation and nodulation stage-specific transcription of the *acdS* gene of *M. loti* MAFF303099 under symbiotic conditions. The ACD-GUS construct clearly demonstrated that *acdS* is exclusively transcribed in bacteroids (Fig. 2). Consistent with the other results, quantitative real-time RT-PCR analysis showed that *acdS* transcription occurred in roots with mature nodules at 10 DAI (Fig. 4) but not in roots at 5 DAI (data not shown), which lacked mature nodules. These results are also consistent with our previous report which showed that the level of transcription of the *acdS* gene was higher under symbiosis conditions than in free-living cells (33).

Using *nifA* mutants, we also demonstrated that the transcriptional regulation of the *acdS* gene of *M. loti* MAFF303099 is dependent on a functional *nifA2* gene under symbiotic conditions (Fig. 4). This result is consistent with the DNA sequence upstream of *acdS*, which contains two −24/−12 type promoters (5) and one upstream activating sequence. The *acdS* gene is present in many soil bacteria, and this gene is known to be regulated mainly by an LRP-like protein, which is an important regulator for amino acid metabolism and related processes (2), even in rhizobia (16, 19). Therefore, the mature nodule-specific and *nifA*-dependent mechanism of *acdS* regulation in *M. loti* is quite different from the mechanism of *acdS* regulation in other bacteria. To the best of our knowledge, this is the first report of the molecular mechanism of symbiosis-specific suppression of ethylene biosynthesis leading to enhancement of rhizobial nodulation.

M. loti acdS seems to have acquired symbiosis-specific functions during its coevolution with the host legume *L. japonicus*. Two physiological effects of ethylene biosynthesis suppressers, such as ACC deaminase and rhizobitoxine, in the establishment of symbiosis have been proposed. One of these effects is enhancement of early infection events on host roots (for reviews see references 24 and 31). The other effect is a positive effect on the establishment and/or maintenance of mature nodules (7, 23). In *Glycine max*, ACC deaminase protein was reported to accumulate in the bacteroid (12). In canola roots, ACC deaminase-producing *E. cloacae* changed the expression of host genes involved in the defense response and cell division (14). Moreover, ethylene application suppressed nitrogenase activity in legume nodules (9). Taken together, the data show that although the detailed physiological functions of ACC deaminase in nodule bacteroids are not clear, ACC deaminase may control the host defense response, mitosis, and nitrogenase activity by lowering the ethylene level around the bacteroids.

Duplicated *nifA* genes. Since two *nifA* genes were found in the *M. loti* MAFF303099 genome, we analyzed the accumulation of *acdS* and *nifH* transcripts in each *nifA* mutant. *nifA2*-

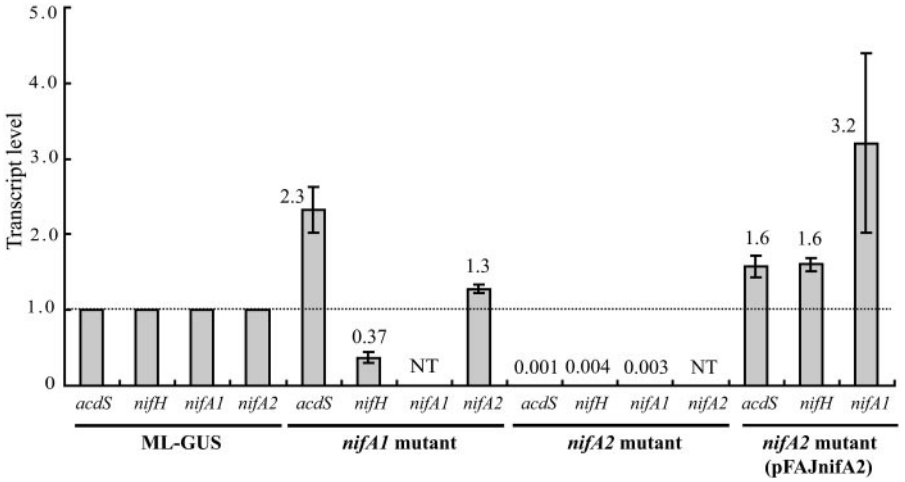

FIG. 4. Quantitative real-time RT-PCR analyses for the *acdS*, *nifH*, *nifA1*, and *nifA2* genes in *M. loti* MAFF303099 for roots at 10 DAI. The relative transcript level for each target gene was normalized to the *gusA* copy number, and the transcript levels for ML-GUS were defined as 1.0. The values are means of three independent experiments, and the error bars indicate standard errors. NT, not tested.

dependent *nifH* regulation suggests that *nifA2* encodes the canonical NifA protein, which may regulate other *nif* genes by collaborating with RNA polymerase σ^{54}, similar to NifA proteins of other nitrogen-fixing bacteria (4). In contrast, regulation by *nifA1* is complicated, and NifA1 may have roles different from those of NifA2 (Fig. 3 and 4). It is interesting that *nifA1* is located in a gene cluster consisting of *nif* and *fix* genes (*nifS*, *nifW*, *fixA*, *fixB*, *fixC*, *fixX*, *nifA1*, *nifB*) (Fig. 1B), and this structure is similar to that of *R. leguminosarum* bv. viciae (20) and the symbiosis island in *M. loti* R7A (32). On the other hand, *nifA2* is located 18 kb from *nifA1*, and it seems to be unaccompanied by other *nif* and *fix* genes. These gene structures suggest that *nifA1* is the original *nifA* gene in *M. loti*.

Two bacteria other than *M. loti* have been reported to possess two *nifA* genes. A nonsulfur phototropic purple bacterium, *Rhodobacter capsulatus*, has two functional copies of *nifA* encoding NifA proteins. However, the amino acid identities and functions of the two NifA proteins are almost the same (26, 27). In *Frankia* sp. strain EuIK1, which forms nitrogen-fixing nodules on an actinorhizal plant, *Elaeagnus umbellatata*, *nifA1* encodes a typical NifA protein, whereas the NifA encoded by *nifA2* is rather similar to AnfA, the alternative nitrogen fixation regulator (18). Our findings and previous reports suggest that symbiotic interactions with host plants may have led to differentiation of the duplicated *nifA* genes.

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