Identification and Functional Analysis of Type III Effector Proteins in *Mesorhizobium loti*

Shin Okazaki,¹ Saori Okabe,¹ Miku Higashi,¹ Yoshikazu Shimoda,² Shusei Sato,² Satoshi Tabata,² Masatsugu Hashiguchi,³ Ryo Akashi,³ Michael Göttfert,⁴ and Kazuhiko Saeki¹

¹Department of Biological Sciences, Faculty of Science, Nara Women's University, Nara 630-8506, Japan; ²Kazusa DNA Research Institute, 2-6-7 Kazusa-kamatari, Kisarazu, Chiba 292-0818, Japan; ³Frontier Science Research Center, University of Miyazaki, 1-1, Gakuen Kibanadai-nishi, Miyazaki-shi, 889-2192, Japan; ⁴Institute of Genetics, Dresden University of Technology, Helmholtzstraße 10, 01069 Dresden, Germany

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Mesorhizobium loti MAFF303099, a microsymbiont of the model legume *Lotus japonicus*, possesses a cluster of genes (*tts*) that encode a type III secretion system (T3SS). In the presence of heterologous *nodD* from *Rhizobium leguminosarum* and a flavonoid naringenin, we observed elevated expression of the *tts* genes and secretion of several proteins into the culture medium. Inoculation experiments with wild-type and T3SS mutant strains revealed that the presence of the T3SS affected nodulation at a species level within the *Lotus* genus either positively (*L. corniculatus* subsp. *frondosus* and *L. filicaulis*) or negatively (*L. halophilus* and two other species). By inoculating *L. halophilus* with mutants of various type III effector candidate genes, we identified open reading frame *mlr6361* as a major determinant of the nodulation restriction observed for *L. halophilus*. The predicted gene product of *mlr6361* is a protein of 3,056 amino acids containing 15 repetitions of a sequence motif of 40 to 45 residues and a shikimate kinase-like domain at its carboxyl terminus. Homologues with similar repeat sequences are present in the hypersensitive-response and pathogenicity regions of several plant pathogens, including strains of *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Xanthomonas* species. These results suggest that *L. halophilus* recognizes *mlr6361* as potentially pathogen derived and subsequently halts the infection process.

Type III secretion systems (T3SSs) are essential for the pathogenicity of many animal- and plant-pathogenic bacteria. Bacteria use the T3SS to deliver effector proteins directly into eukaryotic cells or the external environment, where they manipulate host cellular processes to promote pathogenicity (Grant et al. 2006; Block et al. 2008). Phytopathogenic bacteria often use their effector proteins to suppress host immune responses that are activated by so-called pathogen-associated molecular patterns (PAMPs) (Jones and Dangl 2006). To counteract these threats, host plants have evolved to produce resistance (R) proteins which recognize the incoming effector proteins and allow them to induce resistant responses, including the hypersensitive response (HR) that halts pathogen invasion and disease (Abramovitch et al. 2006; Chisholm et al. 2006).

The genes encoding T3SSs have also been identified in some rhizobia such as *Rhizobium* sp. strain NGR234 (Freiberg et al. 1997), *Sinorhizobium fredii* USDA257 (Krishnan et al. 2003), *S. fredii* HH103 (Marie et al. 2001; de Lyra et al. 2006), *Bradyrhizobium japonicum* USDA110 (Krause et al. 2002), and *Mesorhizobium loti* MAFF303099 (Kaneko et al. 2000). In contrast, *S. meliloti* 1021 (Galibert et al. 2001), *Rhizobium leguminosarum* bv. *viciae* 3841 (Young et al. 2006), and *M. loti* R7A (Sullivan et al. 2002) do not possess T3SS genes. The gene clusters for the T3SS (*tts*) in rhizobia consist of genes encoding structural core components of the T3SS apparatus, secreted proteins, and a transcriptional activator TtsI that induces the expression of other *tts* genes by binding to *cis* elements termed *tts* boxes (Krause et al. 2002; Wassem et al. 2008; Zehner et al. 2008). A *nod* box is located upstream of every rhizobial *ttsI* gene investigated to date. In the presence of compatible flavonoids released from legume roots, activated NodD binds *nod* boxes and activates the expression of nodulation (*nod*) genes. Hence, expression of *ttsI* is controlled by the same regulatory mechanism that controls the biosynthesis of Nod factors, which initiate nodule organogenesis in host legumes (Krause et al. 2002; Marie et al. 2004).

The T3SS can positively or negatively affect symbiosis depending on the host plant. In *B. japonicum*, deletion of *tts* genes causes a reduction in nodule number on *Macroptilium atropurpureum* (Siratro) and delayed nodulation with *Glycine max* (soybean) (Krause et al. 2002). In *S. fredii* USDA257, the T3SS prevents the soybean cv. McCall from being infected (Meinhardt et al. 1993; Bellato et al. 1997). Inactivation of the T3SS affects the ability of *Rhizobium* sp. strain NGR234 to nodulate a variety of tropical legumes (Viprey et al. 1998). The host-dependent symbiotic phenotypes could be caused by the type III effector proteins injected into the host cells. Secreted proteins of rhizobia are designated nodulation outer proteins (Nops) (Marie et al. 2001). The Nops of *Rhizobium* sp. strain NGR234 are either components of the T3SS-dependent pilus (NopA and NopB), the putative translocon that forms pores in the plant plasma membrane (NopX), or type III effectors (NopL, NopP, NopJ, NopM, and NopT), which are secreted via the T3SS (Viprey et al. 1998; Ausmees et al. 2004; Marie et al. 2004; Deakin et al. 2005; Saad et al. 2005; Skorpil et al. 2005; Dai et al. 2008; Kambara et al. 2009).

Mesorhizobium loti is the general name for the microsymbionts of *Lotus* spp. that include the model legume *Lotus japonicus*. Only a few strains, including MAFF303099, had been originally isolated from *L. japonicus* (Saeki and Kouchi 2000). The *M. loti* MAFF303099 genome contains the *tts* gene cluster,

Corresponding author: K. Saeki; Telephone and Fax: +81-742-20-3028; E-mail: ksaeki@cc.nara-wu.ac.jp

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which encodes structural core components of the T3SS apparatus, T3SS-dependent pilus, and TtsI, which is highly similar to those of other rhizobia such as *Rhizobium* sp. strain NGR234 and *B. japonicum* USDA110 (Kaneko et al. 2002). Interestingly, *M. loti* R7A lacks the corresponding genes but contains genes not present in MAFF303099 that encode the type IV secretion system (T4SS). Hubber and associates (2004) reported that both R7A T4SS mutants and a MAFF303099 T3SS mutant nodulated *Leucaena leucocephala*, unlike the wild-type strains. These same mutant phenotypes with *L. leucocephala* suggest that the T3SS of MAFF303099 and the T4SS of R7A play an analogous role in the establishment of the symbiosis. The MAFF303099 genome does not encode the effector proteins identified in NGR234 such as NopL, NopP, NopJ, NopM, and NopT. Instead, MAFF303099 possesses Mr6316, a member of the C48 family of cysteine proteases. Mutation of *mlr6316* resulted in the formation of a small number of nodules on a small proportion of *L. leucocephala* plants, unlike the T3SS structural mutant that effectively nodulated on all inoculated plants (Hubber et al. 2004). Thus, MAFF303099 might secrete other effector proteins that affect the interaction with *L. leucocephala* and other host plants.

In this study, we characterized the T3SS of *M. loti* MAFF303099 by transcriptional and extracellular protein analyses. We also screened several *Lotus* spp. and identified some that exhibited T3SS-dependent symbiotic phenotypes. Using one of these host plants, *Lotus halophilus*, we identified a novel type III effector protein, Mlr6361, that negatively affects the symbiotic interaction.

RESULTS

Genetic organization and transcriptional regulation of the type III secretion system in *M. loti* MAFF303099.

The genes that encode the T3SS in *M. loti* MAFF303099 are clustered within a 25-kb region of the symbiosis island (Fig. 1). This region contains the genes that are conserved in all described *tts* gene clusters of other rhizobia; therefore, we assume that this region encodes all the essential proteins of the secretion machinery. The *tts* gene cluster includes *tssI*, *Rhizobium*-conserved (*rhc*) genes, and *nop* genes that encode puta-

tive secreted proteins. Open reading frames (ORF) encoding putative secreted proteins in the region were *nopA*, *nopB*, *nopC*, and *nopX*. Other known *nops*, such as *nopL*, *nopM*, *nopP*, *nopJ*, and *nopT*, were not found in *M. loti*.

A *nod* box consensus sequence was identified 315 bp upstream of *ttsI*. Seven *tts* box motifs were found in the *tts* region (Figs. 1 and 2). To analyze the expression of the *tts* genes, translational fusions of *lacZ* with *ttsI* and *nopX* were constructed and integrated into the chromosome of *M. loti* strains to yield ML033 and ML031, respectively. To these strains, we subsequently transferred the plasmid pMP2112, which harbors *nodD* from *R. leguminosarum*, (López-Lara et al. 1995) and which had been proven to induce Nod factor synthesis by addition of the flavonoid naringenin in MAFF303099 (Niwa et al. 2001). β -Galactosidase activity in ML033 (pMP2112) increased approximately 13-fold after application of naringenin (Table 1). Similarly, β -galactosidase activity in ML031 (pMP2112) increased approximately eightfold in the presence of naringenin; however, elevated β -galactosidase activity was not observed in response to naringenin in the mutant ML037 which lacks *ttsI*. These results suggested that the *tts* gene cluster in *M. loti* is

CCGTCAGCTTGTGCTGAAGCCAACCGCCTA - 295 bp mlr6316
AGGTCAGAGTCACGTCAGGTGAAGGGGATA - 49 bp mlr6331
CAGTCAGCTTGTGCTAGCTCGGGCCACCTA - 71 bp mlr8763 (nopB)
CCGTCAGCTTAATCGTCAGCCAACGGATCTA - 218 bp mlr6361
TCGTCAGGTTTCTCGAAAGCTCTCTGCTGTA - 57 bp mlr8768 (nopC)
TCGTCAGTTTACCGAAGACTAAACGCTCA - 100 bp mlr6337 (nopX)
GCGTCAGCTCACCCTAGCTCGTTTCGAGTA - 417 bp mlr6358
.cGTCAGett.tCGtcAGet.a.ccgc.tA consensus

tcGTCAGett.tGaaAGet..gcc.c.ta *Rhizobium* sp. NGR234
tcGTCAGetT.tGaaAGet...cc.cctA *B. japonicum* USDA110

Fig. 2. *tts* Box sequences identified in the *Mesorhizobium loti* MAFF303099. Nucleotides conserved in all cases are shown in bold uppercase letters. Numbers indicate the distance in base pairs between the *tts* box and the potential translational start site of the corresponding gene. In the consensus sequence, capital letters are used for invariant nucleotides and lower-case letters are used for nucleotides conserved in at least 50% of the sequences. Consensus sequences from *Rhizobium* sp. strain NGR234 (Marie et al. 2004) and *Bradyrhizobium japonicum* USDA110 (Krause A et al. 2002) are also shown.

Fig. 1. Genetic organization of the *tts* genes of *Mesorhizobium loti* MAFF303099. The 47.4-kbp chromosomal region encompassing the RhizoBase genome coordinates 5138800 and 5186200 is shown. Orientations and sizes of the predicted open reading frames (ORF) are indicated. Gene designations of similar sequences in *Rhizobium* sp. strain NGR234 are given above the ORF. Deletions in mutants DT3S and ML6334 are indicated by broken lines. The direction of the arrows shows the orientation of the inserted kanamycin-resistant cassette. The position of the *nod*-box upstream of *ttsI* is shown by a gray arrowhead and the putative *tts* boxes are shown by unfilled arrowheads.

regulated by NodD, plant-derived flavonoid inducer, and TtsI, as reported in other rhizobia (Krause A et al. 2002, Marie et al. 2004).

Identification of the proteins secreted by the T3SS.

Extracellular proteins from naringenin-induced and uninduced cultures of the wild-type MAFF303099 and type III mutant DT3S containing pMP2112 were partially purified and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Several protein bands were only detected in the induced MAFF303099 cultures (Fig. 3). The molecular weights of these proteins were estimated at approximately 70, 40, 36, 25, 23, 19, 15, and 7 kDa. These proteins were not detected in cultures of the type III mutant DT3S (pMP2112) in either the presence or absence of naringenin (Fig. 3).

The proteins from these bands were excised from the gel and analyzed by mass spectrometry. Significant data were obtained for two proteins of approximately 70 and 15 kDa (Fig.

3, asterisks). The obtained data indicated that the trypsin peptides derived from the band at 70 kDa matched the internal sequences of Mll6337 of *M. loti* MAFF303099, which showed 72% identity with NopX (formerly NolX) of both *Rhizobium* sp. strain NGR234 (NP444155) and *S. fredii* HH103 (AAB17674) (Table 2). Similarly, the peptides derived from the band at 15 kDa matched to the internal sequences of Mlr8763 of *M. loti* MAFF303099, which showed 63% identity with NopB (formerly NolB) of both *Rhizobium* sp. strain NGR234 (NP444157) and *S. fredii* USDA257 (P33208) and 42% identity with NopB (Blr1812) of *B. japonicum* USDA 110 (NP 768452). These results suggested that *M. loti* MAFF303099 encodes a functional T3SS that secretes proteins, including known type III-secreted proteins.

Symbiotic phenotypes of T3SS mutants on *Lotus* plants.

In order to explore the significance of the *M. loti* T3SS, we compared symbiotic phenotypes of wild-type and T3SS mutants on several *Lotus* spp. We constructed a deletion mutant,

Table 1. Transcriptional activation of *ttsI* and *nopX* in *Mesorhizobium loti*

Strain	Genotype	Plasmid pMP2112	β -Galactosidase activity ^a	
			Naringenin + ^b	–
ML031	<i>nopX::lacZ</i>	–	32.9 ± 12.5	31.2 ± 5.3
ML031	<i>nopX::lacZ</i>	+	507.9 ± 84.5	63.2 ± 7.9
ML033	<i>ttsI::lacZ</i>	+	494.9 ± 83.8	36.6 ± 9.2
ML037	<i>nopX::lacZ, ΔttsI</i>	+	42.2 ± 11.0	29.3 ± 10.5

^a Calculated as pmol 4-methylumbelliferone at an optical density at 600 nm per minute of reaction time.

^b Naringenin was added at 1 μM.

Fig. 3. Secreted proteins that are dependent on the type III secretion system (T3SS) in *Mesorhizobium loti* MAFF303099. MAFF303099 and DT3S harboring pMP2112 were cultured in the absence (–) or presence (+) of 1 μM naringenin. Extracellular proteins were concentrated and loaded onto **A**, 12% Tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or **B**, 15% Tris-tricine SDS-PAGE. The molecular weights of protein standards are indicated vertically. Arrowheads and asterisks indicate protein bands that were only visible in the induced culture of MAFF303099 (pMP2112). Asterisks indicate protein bands subjected to mass spectrometry analysis.

DT3S, which lacked an 11-kbp region of the chromosome spanning the *M. loti* MAFF303099 genome coordinates 5,157,472 to 5,168,624, removing 12 conserved genes encoding both structural components of the T3SS and putative secreted proteins (Fig. 1). In the first screening, we identified six species that exhibited discernable differences in nodule number between the wild-type MAFF303099 and type III mutant DT3S. Depending on the species, the presence of the T3SS affected nodulation either positively (*L. corniculatus* subsp. *frondosus* and *L. filicaulis*) or negatively (*L. halophilus*, *L. peregrinus* var. *carmeli*, and *L. subbiflorus*) (Fig. 4). We did not observe any significant difference in nodule number for *L. japonicus* Gifu B-129, although the rate of root elongation increased significantly in response to inoculation of DT3S compared with MAFF303099 (data not shown).

We further analyzed the symbiotic phenotypes of *L. corniculatus* subsp. *frondosus* and *L. halophilus* by inoculating them with the *ttsI* mutant ML6334 and DT3S harboring the cosmid 252.5, which complements the deleted chromosomal region (Fig. 1). On *L. corniculatus* subsp. *frondosus*, the wild-type strain induced more nodules than DT3S (Fig. 4). The phenotype of strain DT3S was partially restored by complementation with cosmid 252.5. Inoculation with ML6334 (Δ *ttsI*) resulted in reduction of nodule numbers to levels similar to those observed for DT3S. Plant growth appeared to be correlated with nodule number (Fig. 5A). Plants were harvested at 45 days after inoculation and fresh weights were determined from at least eight plants per treatment. Plant fresh weights (average $\pm$ standard deviation) were as follows: 521 $\pm$ 91.6 mg with MAFF303099, 303.2 $\pm$ 52.8 mg with DT3S, 576.9 $\pm$ 120.6 mg with DT3S (cosmid 252.5), and 322.8 $\pm$ 52.7 mg with ML6334. In contrast, the presence of T3SS negatively affected nodulation of *L. halophilus* (Fig. 4). This species formed very few nodules upon inoculation with the wild-type strain. DT3S and ML6334 induced more nodules than MAFF303099 and promoted plant growth (Fig. 5B).

Identification of the effector proteins responsible for the symbiotic interaction.

In *Rhizobium* sp. strain NGR234 and *B. japonicum* USDA110, most operons encoding type III-secreted proteins are preceded by a *tts* box motif (Marie et al. 2004; Süss et al. 2006). In the *M. loti* MAFF303099 genome, three of the seven *tts* box motifs were identified upstream of *mlr6337* (*nopX*), *mlr8763* (*nopB*), and *mlr8768* (*nopC*); the products of these are considered components of type III secretion-associated pili, which are required for the injection of effector proteins into the plant cells (Saad et al. 2007). Therefore, we selected the other four ORF that were preceded by *tts* boxes (*mlr6316*, *mlr6331*, *mlr6358*, and *mlr6361*) as type III effector candidates. Transposon mutants of these ORF were isolated from

the signature-tagged mutant library constructed by Shimoda and colleagues (2008). These mutants were inoculated onto *L. corniculatus* subsp. *frondosus* and *L. halophilus*, and the symbiotic phenotypes were compared with the wild-type strain. On *L. corniculatus* subsp. *frondosus*, we could not observe significant differences between wild-type and mutant strains (Fig. 4D). However, on *L. halophilus*, inoculation with ML6361 resulted in the formation of effective nodules, similar to those found following inoculation with DT3S (Figs. 4E and 5B). The plants inoculated with the other mutants were similar to those inoculated with the wild-type strain. These results indicated that the protein encoded by *mlr6361* was responsible for the nodulation restriction by *L. halophilus*.

Features of the *M. loti* type III effector protein Mlr6361.

The predicted product of *mlr6361* is a protein of 3,056 amino acids that contains regions similar to portions of Mlr6331 and to proteins encoded in the hypersensitive-response and pathogenicity (*hrp*) regions found in a number of plant-pathogenic bacteria, including strains of *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Xanthomonas* spp. A phylogenetic tree constructed by the neighbor-joining method using an alignment shown in Supplementary Figure 1 revealed that Mlr6361 and Mlr6331 share more similarity with the *Ralstonia* proteins than with the *Pseudomonas* and *Xanthomonas* proteins (Fig. 6).

Mlr6361 and homologues contain a conserved multidomain “PRK09169” with unknown function found in the conserved domain database. The multidomain corresponds to a large part, approximately residues 800 to 2,800, of Mlr6361. Although PRK09169 (or a portion of PRK09169) is found in a wide range of proteins, its function is unknown and most PRK09169-containing proteins are assigned as hypothetical proteins. The central region of Mlr6361 (residues 916 to 1,572, corresponding to the amino-terminal portion of PRK09169) contains a repeated sequence domain. This repeat domain is composed of 15 repetitions of a sequence motif of 40 to 45 residues that are similar to one another (Fig. 7). Similar repeat domains are also present in Mlr6331 and in homologues located in plant-pathogenic bacteria. The number of repeats ranges from 12 (RSp1374; *R. solanacearum* GMI1000; with GenBank accession number NP_522933) to 26 (XCV4314; *Xanthomonas campestris* pv. *vesicatoria* 85-10; YP_366045).

Comparison of the amino acid sequence of the carboxyl-terminal region of Mlr6361 (residues 2,830 to 3,056) revealed that it was highly similar to shikimate kinase (SK) (EC 2.7.1.71). SK catalyzes the transfer of a phosphoryl group from ATP to shikimate to yield shikimate 3-phosphate. This reaction is the fifth step in the shikimate pathway, whose final product, chorismic acid, is an essential intermediate for the synthesis of aromatic compounds such as aromatic amino acids, folate, and

Table 2. Naringenin-induced secreted proteins in *Mesorhizobium loti* MAFF303099

Band	Estimated molecular mass (kDa)	Proteins identified	MOWSE score ^a	Molecular mass (kDa)	Peptides matched ^b
1	70	Mlr6337 (NopX)	348	68.1	KFFGSGVLTRD KTALEEEAAAAGIDLPI ^M AKS KTHAPETAAEQDAVSDMMTGRA KTHAPETAAEQDAVSD ^{MM} TGRA KAAIEGLQQDP ^{ELFY} AIGSQGDGRC KQAASQLLSDHLLFGLLNNSITGYKT
2	15	Mlr8763 (NopB)	333	17.4	KGVSGITSSVNKL RAATQ ^M SP ^L GD ^R V RVLQTISAMYPDRA RAGSPSFSEQAQFERA

^a MOWSE = Molecular weight search.

^b Oxidized methionine is underlined.

ubiquinone. Sequence alignment with SK family members, including *Mycobacterium tuberculosis* SK (MtSK), of which crystal structures have been extensively investigated (Gu et al. 2002; Gan et al. 2006), revealed that Mlr6361 possesses all the important residues for ATP-binding, in Walker's A- and B-motifs, and for shikimate-binding (Walker et al. 1982; Krell et al. 1998; Gu et al. 2002) (Fig. 7C). Residues equivalent to those proposed to function in a phosphoryl transfer reaction in MtSK (Hartmann et al. 2006) are also located in Mlr6361; such residues include Lys15, Asp34, and Arg117 (in MtSK numbering).

DISCUSSION

In this article, we have described the molecular and genetic analyses of the T3SS in *Mesorhizobium loti* MAFF303099. As in other rhizobia, the expression of the *tts* genes and protein secretion in *M. loti* MAFF303099 was controlled by NodD activating the expression of *ttsI* in the presence of a flavonoid inducer. The fact that expression of the T3SS is coupled to the expression of the *nod* genes ensures that T3SS expression occurs during the infection phase of the symbiotic interaction. It has been reported that NopX of *S. fredii* USDA257 localizes in

Fig. 4. Nodulation of *Lotus* spp. inoculated with wild-type and type III secretion mutants of *Mesorhizobium loti* MAFF303099. Nodule numbers were counted either 45 days (for *Lotus corniculatus* subsp. *frondosus*) or 35 days (the other species) after inoculation. Nodulation tests were performed at least three times, with 12 to 16 plants being tested each time. The values are means obtained from at least 24 plants and the error bars indicate standard deviations. Statistical analyses (Student's *t* test) were performed comparing each mutant to the wild-type strain. A single asterisk represents $P < 0.05$ and double asterisks represent $P < 0.001$.

soybean infection threads but not in mature nodules (Krishnan 2002). We also performed preliminary cytological analysis of the roots of *L. japonicus* inoculated with reporter strains ML031 and ML037 that have chromosomally integrated *lacZ* gene under the control of *nopX* promoter with authentic and disrupted *ttsI* backgrounds, respectively. The coloration derived from β -galactosidase was observed within the infection thread only in the root inoculated with ML031 and not with ML037. In addition, as presented in this study, the abortion of the nodule formation on *L. halophilus* by the wild-type MAFF303099 also suggests that T3SS functions in the infection phase. Animal pathogens utilize T3SS to allow persistence within host cells (Schlumberger and Hardt 2006), while plant pathogens use it to suppress PAMP-triggered innate immunity of host plants (Zhou and Chai 2008). Taken together, it is

likely that the rhizobial T3SS may facilitate release into the symbiosome or suppress host defense response in order to occupy its niche in the infection threads. Recently, Zehner and associates (2008) reported that *B. japonicum* T3SS are expressed in both early infection stages and mature nodules of *M. atropurpureum*. Therefore, it is possible that, in a certain combination of rhizobial species and host legumes, T3SS could also play a role in the later stage of symbiosis.

Our results indicate that *Mr6361* is responsible for the diminished symbiotic interaction with *L. halophilus*. The presence of *Mr6361* homologues has not been reported thus far in rhizobia but some plant pathogens, such as *P. syringae*, *R. solanacearum*, and *Xanthomonas* spp., possess similar genes in their *hrp* region. To date, no evidence has been presented demonstrating that homologous gene products produced by plant

A) *Lotus corniculatus* subsp. *frondosus*

B) *Lotus halophilus*

Fig. 5. *Lotus* spp. plants inoculated with the wild-type strain and *tts* mutants. **A,** *Lotus corniculatus* subsp. *frondosus* inoculated with various strains of *Mesorhizobium loti*, photographed 45 days after inoculation. **B,** *L. halophilus* inoculated with various strains of *M. loti*, photographed 35 days after inoculation.

pathogens are secreted by T3SS or that they possess biological activity in association with host plants. Characteristic features of Mlr6361 include the conserved mutidomain PRK09169 with internal repeat domain and an SK-like domain at its carboxyl terminus. Any or some of these and still unidentified domain might play a role in the host cells, which leads to halting the infection process on *L. halophilus*, as discussed below.

An interesting feature of the conserved domain PRK09169 is the internal repeat sequences (Fig. 7) which is also found in the homologues of phytopathogenic bacteria. The high degree of conservation of the repeat region indicates its important role in defining the structure of the protein. Different types of repeat sequences are found in other type III effectors. Among them, leucine-rich repeats (LRR) are found in a number of type III effectors that are essential for the pathogenicity of animal and plant pathogens (Evdokimov et al. 2001; Haraga and Miller 2003; Zhu et al. 2008; Kambara et al. 2009). The LRR domain is supposed to be involved in protein–protein interactions (Kobe and Kajava 2001). Therefore, there is a possibility that the repeat domain within PRK09169 is involved in interaction with some protein to perturb host functions and recognized by particular hosts like *L. halophilus* to lead to a reaction to halt infection.

Another interesting feature of Mlr6361, the presence of an SK-like domain at its carboxy terminus, suggests that this protein may have an enzymatic function. The shikimate pathway is responsible for the biosynthesis of aromatic compounds in bacteria, yeast, fungi, and plants. In higher plants, the aromatic amino acids produced by this pathway further serve as precursors for various secondary metabolites, such as phenylpropanoids and flavonoids (Herrmann 1995), which have been reported to play important roles in plant defense systems (Dixon and Paiva 1995; Dixon and Steele 1999). Induction of expres-

sion of SK genes by elicitor treatments has been reported in cultured rice cell and tomato cells (Görlach et al. 1995; Kasai et al. 2005). Taken together, there is an alternative possibility that Mlr6361 could act as shikimate kinase in plant cells to perturb host metabolisms to convert a favorable microenvironment for the infection process and that either such function or SK domain itself is recognized by *L. halophilus* to lead to aborted infection. In addition, it might be noted that the homologues of phytopathogenic bacteria do not possess the carboxy-terminus SK domain, although some of them (PSPPH_A0133, RRSL_01097, RRSL_03921, and RRSL_04182) are annotated as shikimate kinases.

Despite the high homology with Mlr6361, Mlr6331 appeared to be unrelated to the nodulation restriction by *L. halophilus*. In addition to the possibility that a feature responsible for the nodulation restriction is present in Mlr6361 but not present in Mlr6331, there is another possibility, that Mlr6331 is not secreted by the T3SS due to an incompatible N-terminal sequence. It has been reported that type III-secreted proteins of *P. syringae* exhibit common features at the N-terminal region: i) high serine percent in the first 50 amino acids, ii) an aliphatic amino acid (Ile, Val, or Leu) or Pro at the third or fourth position, and iii) the absence of negatively charged amino acid in the first 12 amino acids (Guttman et al. 2002; Petnicki-Ocwieja et al. 2002). Because the N-terminal region of Mlr6331 conforms to the features less strictly than that of Mlr6361, it is possible that Mlr6331 is recognized as a less-compatible substrate for T3SS machinery.

In the present study, we could not identify which effector protein of MAFF303099 is responsible for the nodulation enhancement on *L. corniculatus* subsp. *frondosus*. It has been reported that NopL and NopP of *Rhizobium* sp. strain NGR234 are major determinants for the positive effect on symbiosis

Fig. 6. Phylogenetic tree of Mlr6361 and its homologues. A phylogenetic tree was constructed by the neighbor-joining method with the MEGA 4.0 program using full-length amino acid sequence comparison shown in the supplementary figure. GenBank accession numbers are as follows: XOO4401 (*Xanthomonas oryzae* pv. *oryzae* KACC10331), YP_203040; Xoryp_22175 (*X. oryzae* pv. *oryzicola* BLS256), ZP_02245275; PXO_03833 (*X. oryzae* pv. *oryzae* PXO99A), YP_001911573; XOO_4145 (*X. oryzae* pv. *oryzae* MAFF311018), YP_453174; XCV4314 (*X. campestris* pv. *vesicatoria* str. 85-10), YP_366045; XAC4213 (*X. axonopodis* pv. *citri* str. 306), NP_644512; PSPPH_1525 (*Pseudomonas syringae* pv. *phaseolicola* 1448A), YP_273779; PSPPH_A0133 (*P. syringae* pv. *phaseolicola* 1448A), YP_272239; RS05403 (*Ralstonia solanacearum* GMI1000), NP_522491; RSIPO_04310 (*R. solanacearum* IPO1609), YP_002258003; RSp1374 (*R. solanacearum* GMI1000), NP_522933; RRSL_03921 (*R. solanacearum* UW551), ZP_00943380; RSMK02658 (*R. solanacearum* MolK2), YP_002254685; RSc1839 (*R. solanacearum* GMI1000), NP_519960; RSc1839 (*R. solanacearum* GMI1000), CAD15541; hpx30 (*R. solanacearum* RS1000), BAD42408; RSMK01531 (*R. solanacearum* MolK2), YP_002254417; Mlr6361 (*Mesorhizobium loti* MAFF303099), NP_106885; Mlr6331 (*M. loti* MAFF303099), NP_106855; RSp0296 (*R. solanacearum* GMI1000), NP_521857; RRSL_04182 (*R. solanacearum* UW551), ZP_00943200; RSMK02377 (*R. solanacearum* MolK2), YP_002252489; RSc3401 (*R. solanacearum* GMI1000), NP_521520; and RRSL_01097 (*R. solanacearum* UW551), ZP_00946113. The horizontal bar represents a distance of 0.1 substitutions per site.

with tropical legumes *Flemingia congesta* and *Tephrosia vogelii* (Marie et al. 2003; Skorpil et al 2005). Both effectors were shown to be phosphorylated by plant kinases, and NopL could prevent full induction of pathogenesis-related defense proteins,

including chitinase and β -1,3-glucanase, when expressed in plant cells (Bartsev et al. 2004). NopL and NopP are not encoded in the genome of MAFF303099. *M. loti* MAFF303099 secretes several proteins in a T3SS-dependent manner (Fig. 3).

Fig. 7. Features of Mlr6361. **A**, Domain structure of Mlr6361. The three domains of Mlr6361 protein (3,056 amino acids) are illustrated: the N-terminal putative type III secretion signal (first 50 amino acids, filled box); PRK09169 conserved multidomain (residues 803 to 2,794, hatched), with the internal repeat domain (residues 916 to 1,572, vertically striped) consisting of 15 repetitions of a sequence motif of 40 to 45 amino acids; and the C-terminal shikimate kinase-like domain (residues 2,830 to 3,056, gray). The black triangle indicates the insertion point of Ω interposon in mutant ML6361. **B**, Amino acid sequence of the repetitive amino-acid region in the sequence of Mlr6361. The internal region of Mlr6361 (residues 916 to 1,572) is shown. Repeat segments were aligned with the CLUSTAL W algorithm. **C**, Multiple amino acid sequence alignments of a C-terminal shikimate kinase-like domain with other shikimate kinase proteins. The sequences shown include Mlr6361 (NP_106885, residues 2,830 to 3,056), Mlr6331 (NP_106855, residues 2,149 to 2,373), Mll3573 (NP_104650), MtSK (*Mycobacterium tuberculosis* CDC1551 shikimate kinase, AAK46924), and OsSK1 (*Oryza sativa* Japonica group shikimate kinase 1, BAD83412, residues 61 to 295). Walker A and B motifs (boxed) correspond to regions that resemble the functional motifs identified in other nucleotide-binding enzymes (Walker et al. 1982). Two-headed arrows indicate the putative shikimate-binding domain and the LID domain (Gu et al. 2002). Identical residues are in black and similar residues are in gray. Asterisks indicate the residues proposed to function in phosphoryl transfer reaction in MtSK: Lys15, Asp34, and Arg117 in MtSK numbering (Gu et al. 2002; Gan et al. 2006; Hartmann et al 2006).

In the present study, we have identified two of these proteins. Further analysis of the unidentified secreted proteins could provide additional insights into the rhizobial type III effector and its role in legume–*Rhizobium* spp. symbiosis.

In conclusion, we have presented evidence that *M. loti* MAFF303099 has a functional T3SS that is involved in modulation of symbiotic performance with certain host *Lotus* spp. We also showed that the novel type III effector Mlr6361, which has homologues that are widely present in plant pathogens, is a major determinant of nodulation restriction by some host legumes. This is the first genetic evidence that a protein of this group has a biological effect in plant–microbe interactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 3. *M. loti* cultures were grown aerobically at 28°C in tryptone-yeast extract (TY) medium (Hattori et al. 2002) or *Rhizobium*-defined medium (RDM) (Ronson et al. 1987). *Escherichia coli* cells were grown at 37°C in Luria broth or Terrific broth (Sambrook and Russel 2001). Antibiotics were added to the medium at the following concentrations: for *M. loti*, phosphomycin at 100 µg ml⁻¹, tetracycline at 10 µg ml⁻¹, kanamycin at 20 µg ml⁻¹, and spectinomycin at 100 µg ml⁻¹; for *E. coli*, tetracycline at 12.5 µg ml⁻¹, ampicillin at 100 µg ml⁻¹, and kanamycin at 50 µg ml⁻¹.

cin at 20 µg ml⁻¹, and spectinomycin at 100 µg ml⁻¹; for *E. coli*, tetracycline at 12.5 µg ml⁻¹, ampicillin at 100 µg ml⁻¹, and kanamycin at 50 µg ml⁻¹.

Construction of mutants.

Mutants were constructed by a modification of the one-step method described by Datsenko and Wanner (2000) as described previously (Okazaki et al. 2007). Disruption of *ttsI* (mlr6334) was carried out as follows. The F₁ recognition target (FRT)-flanked kanamycin resistance gene (FRT-*kan*-FRT) was polymerase chain reaction (PCR) amplified using pKD13 (Datsenko and Wanner 2000) as the template and primers mlr6334U and mlr6334L, which contained 45-bp extensions homologous to regions flanking mlr6334 (Table 3). The resulting PCR products were electroporated into *E. coli* DH10B harboring both cosmid 251 and plasmid pKD46 and were integrated into the cosmid via Lambda Red-mediated recombination. The cosmid derivatives with deletion insertion loci were transferred to *M. loti* MAFF303099 by conjugation. The deletion mutant ML6334 was obtained as a result of homologous recombination facilitated by plasmid incompatibility using pKS808 as previously described (Hattori et al. 2002). DT3S, which carries an 11-kbp deletion in the *tts* gene cluster spanning 12 genes (mlr6342 to mlr8765) was similarly constructed; in this case,

Table 3. Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Characteristics or sequence ^a	Reference or source
Bacterial strains		
<i>Mesorhizobium loti</i>		
MAFF303099	Wild-type stain; Pm ^r	Saeki and Kouchi 2000; Kaneko et al. 2000
ML6334	Δmlr6334::kan derivative of MAFF303099; Pm ^r , Km ^r	This study
DT3S	MAFF303099 derivative with genome deletion at positions 5,157,472 to 5,168,624 (Δmlr6342-mlr8765) and inserted Km cassette; Pm ^r , Km ^r	This study
ML031	<i>nopX</i> (mlr6337)::lacZ integrated into MAFF303099; Pm ^r , Tc ^r	This study
ML033	<i>ttsI</i> (mlr6334)::lacZ integrated into MAFF303099; Pm ^r , Tc ^r	This study
ML037	<i>nopX</i> (mlr6337)::lacZ integrated into ML6334; Pm ^r , Km ^r , Tc ^r	This study
ML6316	mlr6316::Ω transposon mutant of MAFF303099, 10T03a09 (Mini-Tn insertion at 2,595th nucleotide in total 5247-bp ORF); Pm ^r , Sp ^r	Shimoda et al. 2008
ML6331	mlr6331::Ω transposon mutant of MAFF303099, 11T02g05 (Mini-Tn insertion at 766th nucleotide in total 7122-bp ORF); Pm ^r , Sp ^r	Shimoda et al. 2008
ML6358	mlr6358::Ω transposon mutant of MAFF303099, 40T02g01 (Mini-Tn insertion at 962th nucleotide in total 2439-bp ORF); Pm ^r , Sp ^r	Shimoda et al. 2008
ML6361	mlr6361::Ω transposon mutant of MAFF303099, 27T02d12 (Mini-Tn insertion at 1,605th nucleotide in total 9171-bp ORF); Pm ^r , Sp ^r	Shimoda et al. 2008
<i>Escherichia coli</i>		
DH10B	F ⁻ , <i>mcrA</i> , Δ(<i>mrr-hsdRMS-mcrBC</i>), Ø80d <i>lacZ</i> , ΔM15, Δ <i>lacX74</i> , <i>deoR</i> , <i>recA1</i> , <i>araD139</i> , Δ(<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , λ ⁻ , <i>rpsL</i> , <i>endA1</i> , <i>nupG</i>	Invitrogen
S17-1	<i>recA</i> , <i>pro</i> , <i>hsdR</i> , RP4-2-Tc::Mu-Km::Tn	Simon et al. 1983
Plasmids		
cosmid 251	Ordered cosmid of MAFF303099 containing genome region coordinates 5,133,286-5,161,587; Tc ^r	Hattori et al.
cosmid 252.5	Ordered cosmid of MAFF303099 containing genome region coordinates 5,150,567-5,180,946; Tc ^r	Hattori et al.
pKD13	<i>oriR6K blaM</i> , FRT- <i>aphA</i> -FRT; Km ^r	Datsenko and Wanner 2000
pKD46	<i>oriR101</i> , <i>repA101</i> (Ts), <i>blaM</i> , <i>araBp-gam-bet-exo</i> ; Ap ^r	Datsenko and Wanner 2000
pSUPlacZ481uidA	Derivative of pSUP202 (Simon et al. 1983) used for translational fusions with <i>lacZ</i> and transcriptional fusions with <i>uidA</i> , <i>oriT</i> of RP4, Tc ^r	S. Zehner and M. Göttfert, unpublished
Oligonucleotides		
mlr6334U	5'-CAGTCTCGGTGACCTGTCTACCATTAATAGGAGATGAAATGCGAATTCCGGGGA TCCGTCGACC-3'	This study
mlr6334L	5'-TCGGCCAACTATACGAATGTCGCGCTAGTCACATTCAAGAATGTATGTAGGCTGG AGCTGCTTCG-3'	This study
mlr6342U	5'-GCGTCTCGGCTTCGGGTCGCTTTCTGAAAAAGGCGAACTGTGACAATTCCGGGGAT CCGTCGACC-3'	This study
mlr8765L	5'-AACGCACTCAGGTCCGGACTTCCTTTAAAAACGCAAGCGACAGGTCTGTAGGCTGGA GCTGCTTCG-3'	This study
mlr6334for	5'-AACCGA <u>CTAGT</u> AGGACGACGGTTATC-3'	This study
mlr6334rev	5'-AATAGAGG <u>CCCTC</u> ATTTCATCTCCTA-3'	This study
mlr6337for	5'-TACGT <u>ACTAGT</u> CGCAAAAGAATCAC-3'	This study
mlr6337rev	5'-TTATTAGG <u>CCCTC</u> ATTGTGTCTCTCTCAA-3'	This study

^a Pm^r, Sp^r, Tc^r, Ap^r, and Km^r indicate resistant to phosphomycin, spectinomycin, tetracycline, ampicillin, and kanamycin, respectively. ORF = open reading frame; FRT = F₁ recognition target. Underlines and double underlines indicate restriction sites for *SpeI* and *StuI*, respectively.

the primer pair mlr6342U and mlr8765L (Table 3) was used to amplify the FRT-*kan*-FRT cassette from pKD13. Integration into cosmid 252.5 and subsequent steps for the mutant construction were carried out as described above. The mutants of type III effector candidate genes, including ML6316 (mlr6316::Ωsp), ML6331 (mlr6331::Ωsp), ML6358 (mlr6358::Ωsp), and ML6361 (mlr6361::Ωsp), were isolated by screening a signature-tagged mutant library (Shimoda et al. 2008).

Expression analysis of *tts* genes.

In order to examine the expression of *tts* genes, we constructed *M. loti* strains carrying chromosomally integrated translational *ttsI::lacZ* and *nopX::lacZ* fusion genes (Table 3). To make the *ttsI::lacZ* gene, the upstream 0.55-kbp region of *ttsI*, together with its initiation codon, was amplified by PCR using primers mlr6334for and mlr6334rev (Table 3) from cosmid 251, digested with *SpeI* and *StuI*, and then cloned into the vector pSUPlacZ481uidA (S. Zehner and M. Göttfert, unpublished) that enabled fusion of the coding region of *E. coli lacZ* to the *ttsI* initiation codon. After confirming correct construction by DNA sequencing, the plasmid was transferred to wild-type *M. loti* via biparental conjugation using *E. coli* S17-1. Because the replication origin of the vector does not function in *M. loti*, a strain with the plasmid integrated into the chromosome by single crossover recombination was selected on a TY agar plate containing tetracycline, to which the vector-encoded marker confers resistance, and phosphomycin, to which *M. loti* is naturally resistant. Following confirmation of the correct integration event by PCR, the obtained strain was designated ML033. By similar procedures using primers mlr6337for and mlr6337rev, *nopX::lacZ* fusion strains ML031 and ML037 were constructed with wild-type and Δ*ttsI* backgrounds, respectively.

In order to analyze the transcriptional regulation of *tts* genes under conditions known to induce *nod* genes, we used the heterologous *nod* gene induction system, comprising *nodD* from *Rhizobium leguminosarum* cloned into pMP2112 (López-Lara et al. 1995) and naringenin, because the *nod* gene inducer for *M. loti* has not been identified. The application of naringenin to MAFF303099 strains harboring pMP2112 is reported to induce Nod factor synthesis (Niwa et al. 2001).

Aliquots of RDM minimal medium (20 ml) were inoculated with 200 μl of *M. loti* cultures grown for 48 h to stationary phase. For induction, naringenin was added to a final concentration of 1 μM. Uninduced cultures were treated with the corresponding amount of methanol. Cultures were grown for 48 h at 28°C. β-Galactosidase activity of the cells was then determined as described previously using the 4-methylumbelliferyl-D-galactopyranoside as the substrate (Krause et al. 2002). Fluorescence of the reaction product 4-methylumbelliferone (MU) was measured with a fluorometer (VersaFluor Fluorometer; Bio-Rad Laboratories, Hercules, CA, U.S.A.) by excitation at 365 nm and by emission at 455 nm. Galactosidase activity was calculated as pmol MU at an optical density at 600 nm per minute of reaction time.

Purification and analysis of secreted proteins.

M. loti strains containing pMP2112 were grown in RDM at 28°C for 24 h in the presence or absence of 1 μM naringenin. Cultures were centrifuged at 5,000 × *g* for 30 min and the supernatant was filtered through a membrane with a pore size of 0.45 μm. To precipitate proteins, ammonium sulfate (80% final concentration) was added to the supernatants and the mixture was incubated overnight at 4°C. Proteins were pelleted by centrifugation at 8,000 × *g* for 45 min and resuspended in 50 mM Tris-HCl (pH 7.5). The protein suspension was mixed with trichloroacetic acid (10% wt/vol), incubated on ice overnight,

and centrifuged at 10,000 × *g* for 30 min at 4°C. The protein pellet was washed with ice-cold acetone. After being dried, the pellet was resuspended in 1× SDS sample buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol; pH 6.8). Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories), and the samples were separated on SDS-PAGE (12% wt/vol) gels and silver stained (Yan et al. 2000). To improve resolution of low molecular mass proteins, samples were loaded onto 16% Tricine-SDS-PAGE and prepared as described by Schagger and von Jagow (1987). For mass spectrometry analysis, bands were excised carefully from the gel and sequenced according to Fujiwara and associates (2006). The Mascot database was used to analyze mass spectrometry data (Matrix Science, London).

Plant assays.

Lotus plants used in this study were *L. japonicus* Gifu B-129, *L. filicaulis*, *L. halophilus* (Plant Introduction [PI] accession PI 238336), *L. corniculatus* subsp. *frondosus* (accession PI 283617), *L. peregrinus* var. *carmeli* (accession PI 258396), and *L. subbiflorus* (accession PI 109314). Seed were provided by the Frontier Science Research Center of the University of Miyazaki, Japan, and the United States Department of Agriculture–Agricultural Research Service. For nodulation tests, seed of *Lotus* spp. were scarified, surface-sterilized by immersion in concentrated sulfuric acid for 3 min, rinsed 10 times with sterile water, and germinated on 0.7% (wt/vol) agar plates at 24°C in the dark. After 2 to 3 days, seedlings were transferred to either agar slants made with B&D nitrogen-free medium and 0.9% agar (Broughton and Dilworth 1971) or a plant box (CUL-JAR300; Iwaki, Tokyo, Japan) containing sterile vermiculite watered with B&D nitrogen-free medium. Inoculation of *M. loti* strains and plant cultivation were performed as described previously (Okazaki et al 2007).

Bioinformatic and statistical analyses.

Amino acid sequences were aligned with the CLUSTAL W algorithm following manual adjustment. The phylogenetic tree was constructed by the neighbor-joining method with the MEGA 4.0 program (Tamura et al. 2007). The evolutionary distances were computed in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

National Center for Biotechnology Information Conserved domain database: www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml
 RhizoBase: genome.kazusa.or.jp/rhizobase