Nodule-Specific Cysteine-Rich Peptides Negatively Regulate Nitrogen-Fixing Symbiosis in a Strain-Specific Manner in *Medicago truncatula*

Qi Wang,¹ Jingge Liu,¹ Hua Li,¹ Shengming Yang,¹ Peter Körmöcz,² Attila Kereszt,² and Hongyan Zhu^{1,†}

¹Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546-0312, USA; and ²Institute of Plant Biology, Biological Research Center, Szeged 6726, Hungary

Accepted 3 October 2017.

Medicago truncatula shows a high level of specificity when interacting with its symbiotic partner *Sinorhizobium meliloti*. This specificity is mainly manifested at the nitrogen-fixing stage of nodule development, such that a particular bacterial strain forms nitrogen-fixing nodules (Nod⁺/Fix⁺) on one plant genotype but ineffective nodules (Nod⁺/Fix[−]) on another. Recent studies have just begun to reveal the underlying molecular mechanisms that control this specificity. The *S. meliloti* strain A145 induces the formation of Fix⁺ nodules on the accession DZA315.16 but Fix[−] nodules on Jemalong A17. A previous study reported that the formation of Fix[−] nodules on Jemalong A17 by *S. meliloti* A145 was conditioned by a single recessive allele named *Mtsym6*. Here we demonstrate that the specificity associated with *S. meliloti* A145 is controlled by multiple genes in *M. truncatula*, including *NFS1* and *NFS2* that encode nodule-specific cysteine-rich (NCR) peptides. The two NCR peptides acted dominantly to block rather than promote nitrogen fixation by *S. meliloti* A145. These two NCR peptides are the same ones that negatively regulate nitrogen-fixing symbiosis associated with *S. meliloti* Rm41.

Establishment of a successful symbiotic relationship between legumes and rhizobia requires that the rhizobium can nodulate and invade the host plant and subsequently differentiate and persist in nodule cells to fix nitrogen (Jones et al. 2007; Kereszt et al. 2011; Oldroyd et al. 2011). However, incompatibility between the interacting partners frequently occurs during the process of symbiotic development, leading to a failure of nodulation (Nod[−]) or formation of nodules that are defective in nitrogen fixation (Nod⁺/Fix[−]) (Devine and Kuykendall 1996; Perret et al. 2000; Wang et al. 2012). Genetic control of symbiotic specificity is complex, involving both host and bacterial genes (Wang et al. 2012). Whereas nodulation specificity (i.e., Nod⁺ versus Nod[−]) appears to be mostly controlled by single host genes, nitrogen fixation specificity (i.e., Fix⁺ versus Fix[−]) or natural variation in nitrogen fixation efficiency between different plant-bacteria combinations is quantitatively inherited (Devine and Kuykendall 1996; Parra-Colmenares and Kahn 2005; Yang et al. 2017; Wang et al. 2017). Understanding the molecular mechanisms underlying symbiotic compatibility is important for development of tools to enhance the potential of nitrogen-fixing symbiosis in sustainable agriculture (Devine and Kuykendall 1996).

For most legumes, nodule organogenesis and bacterial infection requires the perception of bacterial lipo-chitooligosaccharides (Nod factors) by the cognate host receptors (Lerouge et al. 1990;

Limpens et al. 2003; Radutoiu et al. 2003, 2007). The specificity of this receptor-ligand interaction relies on the highly variable Nod-factor structures produced by different rhizobia and the polymorphisms of orthologous plant receptors (Radutoiu et al. 2007). Nod factor perception is widely thought to be a major factor that determines nodulation specificity (Lerouge et al. 1990; Perret et al. 2000; Radutoiu et al. 2007). Beyond Nod-factor perception, the symbiotic partners also communicate with each other in a similar manner as pathogenic plant-bacterial interactions, which involves host perception of conserved microbe-associated molecular patterns (MAMP) and bacterial effectors (D’Haeze and Holsters 2004; Deakin and Broughton 2009). Consequently, effector- and MAMP-triggered plant immunity also plays an important role in the regulation of strain-specific nodulation (D’Haeze and Holsters 2004; Deakin and Broughton 2009; Yang et al. 2010; Wang et al. 2012; Kawaharada et al. 2015; Yasuda et al. 2016; Cao et al. 2017).

In contrast to the specificity occurred at early nodulation process, we know much less about the molecular mechanisms that underlie the incompatibility occurred at the nitrogen-fixing phase of nodule development (Wang et al. 2012). Most recently, we isolated two *Medicago truncatula* genes, *NFS1* (nitrogen fixation specificity 1) and *NFS2*, that condition the fixation-level incompatibility concerning *Sinorhizobium meliloti* Rm41 (Yang et al. 2017; Wang et al. 2017). *NFS1* and *NFS2* both encode nodule-specific cysteine-rich (NCR) peptides (Mergaert et al. 2003). In contrast to the known role of NCR peptides as host effectors that mediate bacterial differentiation into nitrogen-fixing bacteroids and maintain bacterial survival (Wang et al. 2010; Van de Velde et al. 2010; Kim et al. 2015; Horváth et al. 2015), we demonstrated that *NFS1* and *NFS2* provoked bacterial cell death and early nodule senescence in an allele-specific and rhizobial strain-specific manner, and their function is dependent on host genetic background (Yang et al. 2017; Wang et al. 2017).

In this report, we present our study on the genetic control of symbiotic specificity involving *S. meliloti* A145 in *M. truncatula*. *S. meliloti* A145 forms Fix[−] nodules on Jemalong A17 but Fix⁺ nodules on other plant genotypes such as DZA315. Tirichine et al. (2000) reported that the formation of Fix[−] nodules on A17 was controlled by a single recessive allele named *Mtsym6*. Here we demonstrate that the specificity associated with *S. meliloti* A145 is controlled by multiple genes, including *NFS1* and *NFS2* that negatively regulate nitrogen-fixing symbiosis with *S. meliloti* Rm41 (Yang et al. 2017; Wang et al. 2017). The two NCR peptides act dominantly to block rather than promote nitrogen fixation by *S. meliloti* A145.

RESULTS AND DISCUSSION

The *M. truncatula* accession DZA315.16 (DZA315) forms Fix⁺ nodules when inoculated with *S. meliloti* A145 (A145), a

[†]Corresponding author: H. Zhu; E-mail: hzhu4@uky.edu.

wild-type strain originally isolated from alfalfa nodules in Syria (Rosenberg et al. 1981; Sun et al. 2006). However, Jemalong A17 (A17) forms Fix^- nodules with this strain even though it forms Fix^+ nodules with many other strains such as *S. meliloti* Rm1021 and *S. medicae* ABS7 (Snyman and Strijdom 1980; Tirichine et al. 2000; Yang et al. 2017). Genetic analysis performed in the segregating populations derived from the cross of Jemalong 6 (Fix^-) and DZA315 (Fix^+) suggested a single recessive allele (named *Mtsym6*) responsible for the Fix^- phenotype on Jemalong 6 (Tirichine et al. 2000). It is noteworthy that Jemalong 6 and Jemalong A17 were both derived from the cultivar Jemalong and did not show any observable genetic and phenotypic differences (Thoquet et al. 2002); therefore, the two lines can be considered as the same genotype. The *Mtsym6* locus was roughly mapped on chromosome 8 (Thoquet et al. 2002), but no further study has been reported ever since.

We reanalyzed the specificity associated with A145 in a set of 71 recombinant inbred lines (RIL) derived from the cross of A17 and DZA315 (Julier et al. 2007). The same RIL lines were recently used by our group for genetic analysis of symbiotic incompatibility associated with *S. meliloti* Rm41 (Rm41) which also forms Fix^+ nodules on DZA315 and Fix^- nodules on A17 (Liu et al. 2014; Yang et al. 2017; Wang et al. 2017). Rm41, originally obtained from alfalfa in Hungary, is closely related to A145 based on multiple gene genealogical analyses (Sun et al. 2006). In both A17/Rm41 and A17/*S. meliloti* A145 interactions, the bacteria colonized the nodule cells and differentiated into elongated nitrogen-fixing bacteroids, but the bacteroids degraded shortly after differentiation, resulting in early nodule senescence (Tirichine et al. 2000; Yang et al. 2017; Wang et al. 2017). We further confirmed the occurrence of bacterial differentiation at early stages of nodule development by inoculating the parental genotypes with an A145 strain carrying a GUS reporter gene driven by the promoter of the *nifH* gene which encodes the iron protein of the nitrogenase enzyme complex. The GUS gene was expressed in the young A17 nodules at one week post inoculation, but the expression was barely detectable three weeks post inoculation due to the lysis of the bacteroids (Fig. 1, A and B). Remarkably, these RILs exhibited highly correlated nitrogen-fixing phenotypes when inoculated with individual A145 and Rm41 strains (Table 1). Among the 71 tested RIL lines, 65 showed similar phenotypes (Phi coefficient $\phi = 0.83$, $P < 0.0001$), simply based on whether or not the plants were capable of forming nitrogen-fixing nodules with the two strains (i.e., Fix^+ or Fix^-) (Yang et al. 2017; Wang et al. 2017). It is noteworthy, however, that there were visually significant differences in their nitrogen fixation efficiency among the RIL lines that were Fix^+ . These observations appeared to suggest that the symbiosis specificities associated with A145 and Rm41 share a common genetic basis, possibly involving the same or linked genes.

We recently reported that the fixation-level incompatibility between A17 and Rm41 was controlled by multiple genes (Yang et al. 2017; Wang et al. 2017). By taking advantage of the two residual heterozygous lines (RHL-NFS1 and RHL-NFS2) identified from the RIL population, we were able to clone two of the underlying genes, namely *NFS1* (*Medtr8g073380*) and *NFS2* (*Medtr8g465280*), both of which encode a nodule-specific cysteine-rich (NCR) peptide (Mergaert et al. 2003). We designated the A17 alleles of *NFS1* and *NFS2* as *NFS1⁻* and *NFS2⁻*, and the DZA315 alleles as *NFS1⁺* and *NFS2⁺* (Yang et al. 2017; Wang et al. 2017). Contrary to the predominant notion that NCR peptides are host effectors that drive bacterioid differentiation required for nitrogen fixation (Wang et al. 2010; Van de Velde et al. 2010; Kim et al. 2015; Horváth et al. 2015; Maróti et al. 2015), we showed that the *NFS1⁻* and *NFS2⁻* alleles acted dominantly to trigger bacterial lysis of Rm41, and both genes function in a context-dependent manner (Yang et al. 2017; Wang et al. 2017). Furthermore, the heterozygous plants (*NFS1^{+/-}* or *NFS2^{+/-}*) exhibited an intermediate phenotype

($\text{Fix}^{+/-}$) in the near-isogenic genetic background of RHL-NFS1 or RHL-NFS2; the $\text{Fix}^{+/-}$ nodules were capable of nitrogen fixation but with lower efficiency when compared with the homozygous Fix^+ genotype.

Our conclusions from the study of the incompatibility of A17 with Rm41 and A145 are in contradiction to the report that the Fix^- phenotype of A17 with A145 was conditioned by a single recessive allele (Tirichine et al. 2000). We suspected that the previous study by Tirichine et al. (2000) failed to realize the variability in nitrogen fixation efficiency among the plants that formed nitrogen-fixing nodules in the segregating populations, and thus classified all those plants as a single Fix^+ group, leading to the conclusion that the Fix^+ phenotype was dominant to Fix^- . Moreover, the underlying genes might be linked with each other on the same chromosome (e.g., *NFS1* and *NFS2*) and appeared to inherit as a single locus in a small segregating population. In fact, we had encountered the same situation when performing genetic analysis of the specificity associated with Rm41, and it took us considerable time to solve the puzzle, thanks to the fortuitous discovery of the RHL-NFS1 and RHL-NFS2 lines (Yang et al. 2017; Wang et al. 2017). To clarify this suspicion, we decided to test whether or not the *NFS1* and *NFS2* genes also regulate symbiotic specificity associated with A145.

We started to address the question by testing the segregation of nitrogen-fixing phenotypes in the self-pollinated progeny of

Fig. 1. Differentiation and persistence of the A145 bacteroids in the DZA315 (Fix^+) and A17 (Fix^-) nodules. **A**, Expression of a β -glucuronidase reporter gene under the control of the *nifH* promoter in the Fix^+ and Fix^- nodules shows that the *nifH* gene was expressed at comparable levels in the Fix^+ and Fix^- nodules at the early phase of nodule development (7 days postinoculation [dpi]), but its expression was abolished at later timepoints (14 and 21 dpi) in the Fix^- nodules. **B**, Micrograph of the bacterial populations isolated from the Fix^+ and Fix^- nodules and stained with the green fluorescent nucleic acid stain SYTO 9, showing the gradual degradation of elongated bacteroids in the Fix^- nodules. Bars = 500 μm in A and 5 μm in B.

the residual heterozygous plants of RHL-NFS1 and RHL-NFS2. In the near-isogenic progeny of RHL-NFS1 inoculated with A145, the segregation of the Fix^- and Fix^+ phenotypes statistically fits the 3:1 ratio (Fix^- to Fix^+), indicating that the Fix^- phenotype is controlled by a single dominant gene in this population (Fig. 2A). In consistent with this observation, the plants homozygous for the $NFS1^+$ allele ($NFS1^{+/+}$) showed the Fix^+ phenotype, while plants homozygous for the $NFS1^-$ allele ($NFS1^{-/-}$) and heterozygous plants ($NFS1^{+/-}$) were Fix^- (Fig. 2, B to H). The Fix^- phenotype of the heterozygous plants with A145 was in contrast to the intermediate phenotype with Rm41 (Yang et al. 2017), suggesting that the $NFS1$ locus show differential effects on the two strains. The $NFS2$ locus showed a similar inheritance pattern, except for that the heterozygous progeny exhibited an intermediate phenotype ($\text{Fix}^{+/+}$) (Fig. 3). These results suggested that both $NFS1$ and $NFS2$ loci are involved in the regulation of strain-specific nitrogen fixation with A145.

We next tested the function of the $NFS1^-$ and $NFS2^-$ alleles by means of complementation tests and CRISPR/Cas9-mediated gene disruption. All the experiments were performed in the near-isogenic background of RHL-NFS1 or RHL-NFS2 through hairy root transformation. As expected, introduction of the A17 allele ($NFS1^-$) into the $NFS1^{+/+}$ background of RHL-NFS1 resulted in the formation of Fix^- nodules on the transgenic roots (Fig. 4, A and B). Consistent with this observation, CRISPR/Cas9-mediated knockout of the $NFS1^-$ allele in the $NFS1^{-/-}$ background of RHL-NFS1 converted the Fix^- phenotype to Fix^+ (Fig. 4, C and D).

These results indicate that $NFS1^-$ acts dominantly to block nitrogen fixation by *S. meliloti* A145. Using the same approaches, we demonstrated in the near-isogenic RHL-NFS2 background that $NFS2^-$ is responsible for developing the Fix^- phenotype by A145 (Fig. 5).

In addition to $NFS1$ and $NFS2$, the specificity associated with A145 also involves other host genes. For instance, certain RIL lines possessing homozygous $NFS1^-$ and/or $NFS2^-$ alleles showed the Fix^+ phenotype, suggesting that the negative role of the Fix^- alleles can be suppressed in these genetic backgrounds (Table 1). In particular, $NFS2^-$ does not negatively affect symbiotic persistence with A145 in the A17 background, because knockout of $NFS1^-$ (but not $NFS2^-$) in A17 led to the formation of Fix^+ nodules (Fig. 6); the same was also true for Rm41 (Wang et al. 2017).

$NFS1$ and $NFS2$ are canonical NCR peptides, each with an N-terminal signal sequence and a putative mature peptide with four ($NFS2$) or six ($NFS1$) conserved cysteine residues (Yang et al. 2017; Wang et al. 2017). There are three amino-acid substitutions that distinguish between $NFS1^-$ (A17) and $NFS1^+$ (DZA315), as well as between $NFS2^-$ and $NFS2^+$ mature peptides. None of these substitutions involve the conserved cysteine residues. When inoculated with A145, both genes are exclusively expressed in root nodules, predominantly in cells around the transition between the infection and fixation zones (Fig. 7, A and B). This expression pattern is similar to that observed in nodules infected by other strains (Roux et al. 2014; Yang et al. 2017; Wang et al. 2017). Transgenic nodules expressing an $NFS1^-$ -mCherry

Table 1. Genotyping and phenotyping of a recombinant inbred line population derived from the cross of A17 and DZA315^a

RIL No.	Genotype		Phenotype		RIL No.	Genotype		Phenotype	
	<i>NFS1</i>	<i>NFS2</i>	Rm41	A145		<i>NFS1</i>	<i>NFS2</i>	Rm41	A145
1	-/-	-/-	Fix^-	Fix^-	84 [†]	-/-	+/+	Fix^+	Fix^+
9	+/+	+/+	Fix^+	Fix^+	86	+/+	+/+	Fix^+	Fix^+
13*	-/-	-/-	Fix^+	Fix^-	92	+/+	+/+	Fix^+	Fix^+
14 [†]	-/-	-/-	Fix^+	Fix^+	98 [†]	-/-	+/+	Fix^+	Fix^+
17	+/+	+/+	Fix^+	Fix^+	100	-/-	-/-	Fix^-	Fix^-
18	+/+	+/+	Fix^+	Fix^+	109	+/+	+/+	Fix^+	Fix^+
21	+/+	+/+	Fix^+	Fix^+	110	+/+	+/+	Fix^+	Fix^+
23	+/+	+/+	Fix^+	Fix^+	127	-/-	+/+	Fix^-	Fix^-
25 [†]	+/+	-/-	Fix^+	Fix^+	131	+/+	+/+	Fix^+	Fix^+
27	+/+	+/+	Fix^+	Fix^+	135 [†]	-/-	+/+	Fix^+	Fix^+
29	-/-	-/-	Fix^-	Fix^-	136	+/+	+/+	Fix^+	Fix^+
32	-/-	-/-	Fix^-	Fix^-	144 [†]	-/-	+/+	Fix^+	Fix^+
35	+/+	+/+	Fix^+	Fix^+	147	-/-	-/-	Fix^-	Fix^-
36 [†]	+/+	-/-	Fix^+	Fix^+	148	+/+	+/+	Fix^+	Fix^+
39	+/+	+/+	Fix^+	Fix^+	157*	+/+	-/-	Fix^+	Fix^-
43	-/-	-/-	Fix^-	Fix^-	159	+/+	+/+	Fix^+	Fix^+
44	-/-	-/-	Fix^-	Fix^-	162	+/+	+/+	Fix^+	Fix^+
45	+/+	+/+	Fix^+	Fix^+	164	-/-	-/-	Fix^-	Fix^-
48	-/-	+/+	Fix^-	Fix^-	182	-/-	+/+	Fix^-	Fix^-
49	-/-	-/-	Fix^-	Fix^-	188	+/+	+/+	Fix^+	Fix^+
50	-/-	+/+	Fix^-	Fix^-	193*	+/+	-/-	Fix^+	Fix^-
51	+/+	+/+	Fix^+	Fix^+	194	-/-	-/-	Fix^-	Fix^-
53	-/-	-/-	Fix^-	Fix^-	196*	-/-	+/+	Fix^+	Fix^-
54	+/+	+/+	Fix^+	Fix^+	197 [†]	+/+	-/-	Fix^+	Fix^+
57 [†]	+/+	-/-	Fix^+	Fix^+	200	+/+	+/+	Fix^+	Fix^+
59 [†]	+/+	-/-	Fix^+	Fix^+	203	-/-	-/-	Fix^-	Fix^-
60	-/-	-/-	Fix^-	Fix^-	209	-/-	-/-	Fix^-	Fix^-
61*	-/-	+/+	Fix^+	Fix^-	213 [†]	-/-	+/+	Fix^+	Fix^+
62	+/+	+/+	Fix^+	Fix^+	225	-/-	-/-	Fix^-	Fix^-
64	+/+	+/+	Fix^+	Fix^+	226	-/-	-/-	Fix^-	Fix^-
65	+/+	+/+	Fix^+	Fix^+	235	-/-	+/+	Fix^-	Fix^-
70	-/-	-/-	Fix^-	Fix^-	236*	-/-	-/-	Fix^+	Fix^-
73	+/+	+/+	Fix^+	Fix^+	238	+/+	+/+	Fix^+	Fix^+
78	+/+	+/+	Fix^+	Fix^+	RHL-NFS1	+/+	+/+	Segregation	Segregation
79	-/-	-/-	Fix^-	Fix^-	RHL-NFS2	-/-	+/+	Segregation	Segregation
80	-/-	-/-	Fix^-	Fix^-					

^a -/- = homozygous A17 alleles, +/+ = homozygous DZA315 alleles, and +/- = heterozygous alleles. The dagger symbol (†) indicates the lines that possess one or both homozygous $NFS1^-$ and $NFS2^-$ alleles but form nitrogen-fixing (Fix^+) nodules with A145. An asterisk (*) indicates lines showing differential phenotypes when inoculated with *Sinorhizobium meliloti* strains Rm41 and A145.

Fig. 2. Segregation of the Fix⁺ and Fix⁻ phenotypes in the progeny of RHL-NFS1. **A**, The 3:1 segregation ratio of the Fix⁻ and Fix⁺ phenotypes in the near-isogenic progeny derived by selfing of the residual heterozygous RHL-NFS1 plants, suggesting a single dominant gene controlling the Fix⁻ phenotype. **B** to **E**, Plants forming the Fix⁺ and Fix⁻ nodules are visually distinguishable by their growth vigor (**B**) and nodule size and color (**C** to **E**). Scale bars = 500 μ m. **F**, Plant dry weight, **G**, nodule dry weight, and **H**, nodule numbers differ significantly between plants forming Fix⁺ and Fix⁻ nodules.

Fig. 3. Segregation of the Fix⁺, Fix^{+/-}, and Fix⁻ phenotypes in the progeny of RHL-NFS2. **A**, The 1:2:1 segregation ratio of the Fix⁺, Fix^{+/-}, and Fix⁻ phenotypes in the near-isogenic progeny derived by selfing of the residual heterozygous RHL-NFS2 plants, suggesting a single gene control of the trait. **B** to **E**, Plants forming the Fix⁺, Fix^{+/-}, and Fix⁻ nodules are visually distinguishable by their growth vigor (**B**) and nodule size and color (**C** to **E**). Scale bars, 500 μ m. **F**, Plant dry weight, **G**, nodule dry weight, and **H**, nodule numbers differ significantly between plants forming Fix⁺, Fix^{+/-}, and Fix⁻ nodules.

Fig. 4. Functional validation of the *NFS1*[−] allele in the regulation of symbiotic specificity associated with A145 in the near-isogenic background of RHL-*NFS1*. **A** and **B**, Expressing the *NFS1*[−] allele in the *NFS1*^{+/+} (Fix^+) background led to the formation of Fix^- nodules on the transgenic roots (indicated by the red arrowheads), whereas the nontransgenic root formed Fix^+ nodules (indicated by the white arrowhead). Images **A** and **B** were the same roots before (**A**) and after (**B**) β -glucuronidase (GUS) staining to indicate the transgenic roots. **C**, CRISPR/Cas9-mediated knockout of the *NFS1*[−] allele in the *NFS1*^{−/−} (Fix^-) background resulted in the formation of Fix^+ nodules on the transgenic roots (indicated by the red arrowheads), whereas the nontransgenic roots formed Fix^- nodules (indicated by the white arrowhead). Insets show GUS staining of the same nodules/roots in the main image, as an indicator for the transgenic roots. **D**, Sequence analysis revealed two mutant alleles in the transgenic root forming Fix^+ nodules shown in **C** with the red box, one with a 5-bp deletion and another with a 4-bp deletion (indicated by arrows), while the roots forming Fix^- nodules are nontransgenic and contained the wild-type A17 allele.

Fig. 5. Functional validation of the *NFS2*[−] allele in the regulation of symbiotic specificity associated with A145 in the near-isogenic background of RHL-*NFS2*. **A** and **B**, Expressing the *NFS2*[−] allele in the *NFS2*^{+/+} (Fix^+) background led to the formation of Fix^- nodules on the transgenic roots (indicated by the red arrowhead), whereas the nontransgenic roots formed Fix^+ nodules (indicated by the white arrowhead). Images **A** and **B** were the same roots before (**A**) and after (**B**) GUS staining to indicate the transgenic roots. **C** and **D**, CRISPR/Cas9-mediated knockout of the *NFS2*[−] allele in the *NFS2*^{−/−} (Fix^-) background resulted in the formation of Fix^+ nodules on the transgenic root (indicated by the red arrowhead), whereas the nontransgenic roots formed Fix^- nodules (indicated by the white arrowheads). Images **C** and **D** were the same roots before (**C**) and after (**D**) GUS staining to indicate the transgenic roots. **E**, Sequence analysis revealed two mutant alleles in the transgenic root forming Fix^+ nodules shown in **C** and **D**, one with a "A" deletion and another with a "T" insertion (indicated by arrows), while the roots forming Fix^- nodules are nontransgenic and contain the wild-type A17 allele.

(Fig. 7, C-E) or *NFS2*⁻-mCherry (Fig. 7, F-H) translational fusion under the control of their native promoter showed that the peptides colocalized with bacteroids in the nodule cells with viable bacteria at two weeks post inoculation.

NCRs have been shown to be essential for mediating bacterial differentiation required for nitrogen fixation in *M. truncatula* (Wang et al. 2010; Van de Velde et al. 2010; Kim et al. 2015; Horváth et al. 2015). Some of these peptides showed bactericidal activities when applied to free-living bacteria; the same was also true for *NFS1*⁻ and *NFS2*⁻ that exhibited antimicrobial activities toward Rm41 in vitro (Yang et al. 2017; Wang et al. 2017). When applied to free-living A145, synthetic *NFS2*⁻ (A17) peptide caused bacterial cell death, as indicated by a significant reduction of colony-forming units in plating assays (Fig. 8A). In contrast, the in vitro bactericidal effect was not apparent for the *NFS2*⁺ (DZA315) peptide. Similarly, induced expression of *NFS1*⁻ in A145 significantly reduced the

bacterial growth rate, as evidenced by the decrease of colony size in plating assays, but the expression of the *NFS1*⁺ peptide showed the similar effect (Fig. 8B). These tests showed that the peptides possess the antibacterial property in vitro but this property is not necessarily correlated with their in planta function due to the involvement of other genes in the genetic background.

CONCLUSION

In *M. truncatula* and closely related galegoid legumes that form indeterminate nodules, the host genomes encode a large number of NCR peptides (Montiel et al. 2017). These NCR genes are almost exclusively expressed in the infected nodule cells and thus are important factors regulating symbiotic development (Mergaert et al. 2003; Roux et al. 2014). NCR peptides apparently can play both positive and negative roles in the establishment of the

Fig. 6. Functional test of the *NFS1*⁻ and *NFS2*⁻ alleles in the A17 background. **A to C**, CRISPR/Cas9-mediated knockout of *NFS1*⁻ in A17 converted the Fix⁻ phenotype to Fix⁺. Images **A** and **B** represented the same root before (**A**) and after (**B**) GUS staining, as an indicator for the transgenic root. Scale bars in **A** and **B**, 500 μm. Sequence analysis identified two mutant alleles in the transgenic root forming Fix⁺ nodules shown in panels **A** and **B**, one with a "C" deletion and another with a 4-bp deletion. **D to F**, CRISPR/Cas9-mediated knockout of *NFS2*⁻ in the A17 background retained the Fix⁻ phenotype. Images **D** and **E** were the same root before (**D**) and after (**E**) GUS staining, as an indicator for the transgenic root. Scale bars in **D** and **E**, 500 μm. Sequence analysis revealed a 2-bp deletion in the transgenic root forming Fix⁺ nodules shown in **D** and **E**.

Fig. 7. Expression and cellular localization of *NFS1* and *NFS2* peptides. **A** promoter-β-glucuronidase assay shows that **A**, *NFS1* and **B**, *NFS2* are predominantly expressed around the transition between the infection and fixation zones in the Fix⁺ nodules inoculated by A145. I, meristem zone; II, infection zone; III, nitrogen fixation zone; IZ, interzone. Scale bars, 200 μm. **C to E**, Localization of GFP-tagged bacteroids (**C**), *NFS1*⁻-mCherry (**D**), and an overlay (**E**) in a symbiotic cell, showing that the *NFS1*⁻ peptide colocalized with the symbiosomes before the bacteroids were completely killed. **F to H**, Localization of GFP-tagged bacteroids (**F**), *NFS2*⁻-mCherry (**G**), and an overlay (**H**) in a symbiotic cell, showing that the *NFS2*⁻ peptide colocalized with the symbiosomes. Scale bars in **C** to **H**, 5 μm.

legume-rhizobial symbiosis that involves terminal bacteroid differentiation (Wang et al. 2010; Van de Velde et al. 2010; Kim et al. 2015; Horváth et al. 2015; Yang et al. 2017; Wang et al. 2017). Both roles are associated with the antimicrobial property of the peptides. On one hand, the host uses this antibiotic strategy for directing terminal bacteroid differentiation to optimize nitrogen fixation and nutrient exchange (Oono and Denison 2010; Oono et al. 2010; Wang et al. 2010; Van de Velde et al. 2010). On the other hand, this study, together with the two recent reports (Yang et al. 2017; Wang et al. 2017), showed that the symbiotic relationship could not sustain if the bacteria cannot survive the antibacterial activity of certain peptide isoforms. The sensitivity of particular bacterial strains in response to a peptide is dependent on the genetic composition of the bacteria as well as the host genetic background. For example, even though the NFS1⁻ peptide functions against both Rm41 and A145, the A145 strain appears to be more sensitive to the peptide because the heterozygote formed Fix^{+/−} nodules with Rm41 but Fix⁻ nodules with A145 (Yang et al. 2017; Wang et al. 2017). We propose that this host-symbioint adaptation drives the coevolution of both symbiotic partners, resulting in the rapid amplification and diversification of the *NCR* genes in galegoid legumes.

MATERIALS AND METHODS

Plant materials and nodulation assay.

The *M. truncatula* RIL population was provided by Dr. Jean-Marie Prosperi (Institut National de la Recherche Agronomique, Montpellier, France). In this study, we used only a subset of the lines that we used in previous studies (Yang et al. 2017; Wang et al. 2017) because of the lack of seeds for other lines when the experiments were performed. Plants were grown in a mixture of vermiculite and Turface in a growth chamber programmed for 16 h light at 22°C and 8 h dark at 20°C. For nodulation assay, roots of one-week-old seedlings were flood-inoculated with *S. meliloti* A145. Plants were watered with half-concentrated Hoagland solution without nitrogen. Nitrogen-fixing phenotypes of nodules were assayed four weeks post inoculation.

Plasmids and vectors.

For complementation tests and CRISPR/Cas9-mediated gene knockouts, we used the same plasmids and vectors as previously described (Yang et al. 2017; Wang et al. 2017). For complementation tests, the genomic constructs of the *NFS1*⁻ and *NFS2*⁻ alleles were cloned into the pCambia1305.1 vector that contained a GUSPlus gene expression cassette. The CRISPR/Cas9 constructs were developed by using the pKSE401 vector described by Xing et al. (2014). To facilitate the identification of transgenic roots, we

amplified the GUSPlus gene cassette from pCambia1305.1 and engineered it into the pKSE401 vector. Two pairs of oligos were designed to specifically target the *NFS1*⁻ and *NFS2*⁻ alleles, respectively. For *NFS1*⁻, we used the oligo pair 5'ATTGTTGAAA GAGGTGTAAGCTC3' and 5'AAACGAGCTTACACCTCTTT CAA3'. For *NFS2*⁻, we used the oligo pair 5'ATTGTTATAA AAGATATCCTCGT3' and 5'AAACACGAGGATATCTTTTAT AA3'. The underlined sequences represent the targeted sites. The oligo pairs were first annealed to produce a double-stranded fragment with 4-nt 5' overhangs at both ends, and then ligated into the *Bsa*I digested pKSE401 vector. The *NFS1*⁻/mCherry and *NFS2*⁻/mCherry translational fusion constructs were developed by in-frame fusion between the second exon of each gene and the mCherry coding sequence in pCambia1305.1. For promoter-GUS assay, the promoter region of *Medtr8g073380* (*NFS1*) or *Medtr8g465280* (*NFS2*) was first cloned into the pDONR/Zeo vector (Invitrogen) and then subcloned into the pMDC163 vector using the Gateway cloning system (Invitrogen).

Hairy root transformation and analysis of transgenic roots.

Plasmids were introduced into the *Agrobacterium rhizogenes* strain ARqua1, and hairy root transformations were performed based on the protocols described by Boisson-Dernier et al. (2001). The transgenic roots were identified by GUS staining. For the CRISPR/Cas9-based gene knockout experiments, the DNA samples were obtained from transgenic roots, which were subjected to PCR amplification of the target sites followed by DNA sequencing to validate the DNA mutations. If the initial sequencing indicated the presence of multiple heterogeneous mutant alleles, we cloned the PCR products into the pGEM T-Easy Vector System (Promega) and more than 10 colonies were selected for sequencing.

Microscopy.

For fluorescence microscopy, nodules infected by GFP-tagged A145 and expressing a mCherry-fused protein were hand-sectioned using double-edged razorblades and mounted on microscope slides in 0.1 M phosphate buffer (pH7.4) containing 25 mg/ml sucrose. Sectioned nodules were examined using a FV1000 confocal laser scanning microscope (Olympus).

Antimicrobial activity assay for the NFS1 and NFS2 peptides.

We used the same methods as described in Yang et al. (2017) and Wang et al. (2017). Antimicrobial activity of the NFS1 peptides was assessed by expressing the *NFS1*⁻ (A17) or *NFS1*⁺ (DZA315) allele in *S. meliloti* A145 through the broad-host-range

Fig. 8. Assay for antibacterial activities of the NFS1 and NFS2 peptides on free-living *Sinorhizobium meliloti* A145. **A**, Bactericidal assay of the synthetic NFS2 peptides on A145, showing that the NFS2⁻ peptide significantly blocked the bacterial proliferation ($P < 0.0001$), whereas the NFS2⁺ peptide had no significant effect on bacterial growth. Data are means $\pm$ standard deviation ($n = 3$). **B**, Assay for antibacterial activity of the NFS1 peptides on A145. The bacteria containing the pSRKtc vector grew at a similar rate with or without the inducer isopropyl β -D-1-thiogalactopyranoside (IPTG). However, induced expression of both NFS1⁺ or NFS1⁻ by IPTG significantly ($P < 0.0001$) decreased the bacterial growth rate, leading to reduced colony sizes.

expression vector pSRKTc (Khan et al. 2008). Expression of the genes was driven by a *lacI^q-lac* promoter-operator complex. The plasmids were mobilized into the rhizobial strain through triparental mating with the helper plasmid pRK600. Cultures of A145 (OD₆₀₀ = 0.4), each harboring pSRKTc, pSRKTc-*NFSI*⁻ or pSRKTc-*NFSI*⁺, were diluted to 10⁻⁶. 100 µl of each dilution was plated on to the agarose plates with TY medium and tetracycline (10 µg/L) in the presence or absence of 4 mM IPTG. The colony size was evaluated after incubation at 30°C for three days.

In vitro NFS2 activities were assayed by using the synthetic peptides. The peptides were synthesized by LifeTein (www.LifeTein.com). Briefly, the bacteria were cultured to OD₆₀₀ of 0.3 in LB media, washed with 5 mM Mes-KOH at pH 5.8, and then diluted to OD₆₀₀ of 0.1 in the same buffer. 200 µl of bacteria were treated with the NFS2⁻ or NFS2⁺ peptides at a series of concentrations and incubated at 30°C for 3 h. Bacterial suspensions were then serially diluted and plated out in triplicate on selective medium. Colony-forming units were counted after 2 days of incubation at 30°C.

ACKNOWLEDGMENTS

We would like to thank Jean-Marie Prosperi (Institut National de la Recherche Agronomique), Laurent Gentzbittel (Université de Toulouse), Peter Mergaert (Université Paris-Sud), Sharon Long (Stanford University), Clay Fuqua (Indiana University), and Qijun Chen (China Agricultural University) for providing materials for this study. This work was supported by the USDA/NIFA's Agriculture and Food Research Initiative (grant number 2014-67013-21573 to H.Z.), the Kentucky Science and Engineer Foundation (grant number 2615-RDE-015 to H.Z.), and the Hungarian National Research Fund/National Research, Development and Innovation Office (OTKA) (grant number 120122/120300 to A.K.). Confocal microscopy work was supported by the US National Science Foundation under Cooperative Agreement No. 1355438.

LITERATURE CITED

Boisson-Dernier, A., Chabaud, M., Garcia, F., Bécarré, G., Rosenberg, C., and Barker, D. G. 2001. *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Mol. Plant-Microbe Interact* 14:695-700.

Cao, Y., Halane, M. K., Gassmann, W., and Stacey, G. 2017. The role of plant innate immunity in the legume-rhizobium symbiosis. *Annu. Rev. Plant Biol.* 68:535-561.

D'Haese, W., and Holsters, M. 2004. Surface polysaccharides enable bacteria to evade plant immunity. *Trends in microbial.* 12: 555-561.

Deakin, W. J., and Broughton, W. J. 2009. Symbiotic use of pathogenic strategies: rhizobial protein secretion systems. *Nat. Rev. Microbiol.* 7: 312-320.

Devine, T. E., and Kuykendall, L. D. 1996. Host genetic control of symbiosis in soybean (*Glycine max* L.). *Plant Soil* 186:173-187.

Horváth, B., Domonkos, A., Kereszt, A., Szűcs, A., Ábrahám, E., Ayaydin, F., Bóka, K., Chen, Y., Chen, R., Murray, J. D., Udvardi, M. K., Kondorosi, É., and Kaló, P. 2015. Loss of the nodule-specific cysteine rich peptide, NCR169, abolishes symbiotic nitrogen fixation in the *Medicago truncatula* *dnf7* mutant. *Proc. Natl. Acad. Sci. U.S.A.* 112: 15232-15237.

Jones, K. M., Kobayashi, H., Davies, B. W., Taga, M. E., and Walker, G. C. 2007. How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. *Nat. Rev. Microbiol.* 5:619-633.

Julier, B., Huguet, T., Chardon, F., Ayadi, R., Pierre, J. B., Prosperi, J. M., Barre, P., and Huyghe, C. 2007. Identification of quantitative trait loci influencing aerial morphogenesis in the model legume *Medicago truncatula*. *Theor. Appl. Genet.* 114:1391-1406.

Kawaharada, Y., Kelly, S., Nielsen, M. W., Hjuler, C. T., Gysel, K., Muszyński, A., Carlson, R. W., Thygesen, M. B., Sandal, N., Asmussen, M. H., Vinther, M., Andersen, S. U., Krusell, L., Thirup, S., Jensen, K. J., Ronson, C. W., Blaise, M., Radutoiu, S., and Stougaard, J. 2015. Receptor-mediated exopolysaccharide perception controls bacterial infection. *Nature* 523:308-312.

Kereszt, A., Mergaert, P., and Kondorosi, E. 2011. Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims? *Mol. Plant-Microbe Interact* 24:1300-1309.

Khan, S. R., Gaines, J., Roop, R. M., 2nd, and Farrand, S. K. 2008. Broad-host-range expression vectors with tightly regulated promoters and their

use to examine the influence of TraR and TraM expression on Ti plasmid quorum sensing. *Appl. Environ. Microbiol.* 74:5053-5062.

Kim, M., Chen, Y., Xi, J., Waters, C., Chen, R., and Wang, D. 2015. An antimicrobial peptide essential for bacterial survival in the nitrogen-fixing symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 112:15238-15243.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C., and Dénarié, J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* 344:781-784.

Limpens, E., Franken, C., Smit, P., Willemse, J., Bisseling, T., and Geurts, R. 2003. LysM domain receptor kinases regulating rhizobial Nod factor-induced infection. *Science* 302:630-633.

Liu, J., Yang, S., Zheng, Q., and Zhu, H. 2014. Identification of a dominant gene in *Medicago truncatula* that restricts nodulation by *Sinorhizobium meliloti* strain Rm41. *BMC Plant Biol.* 14:167.

Maróti, G., Downie, J. A., and Kondorosi, É. 2015. Plant cysteine-rich peptides that inhibit pathogen growth and control rhizobial differentiation in legume nodules. *Curr. Opin. Plant Biol.* 26:57-63.

Mergaert, P., Nikovics, K., Kelemen, Z., Maunoury, N., Vaubert, D., Kondorosi, A., and Kondorosi, E. 2003. A novel family in *Medicago truncatula* consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. *Plant Physiol.* 132:161-173.

Montiel, J., Downie, J. A., Farkas, A., Bihari, P., Herczeg, R., Bálint, B., Mergaert, P., Kereszt, A., and Kondorosi, É. 2017. Morphotype of bacteroids in different legumes correlates with the number and type of symbiotic NCR peptides. *Proc. Natl. Acad. Sci. U.S.A.* 114:5041-5046.

Oldroyd, G. E., Murray, J. D., Poole, P. S., and Downie, J. A. 2011. The rules of engagement in the legume-rhizobial symbiosis. *Annu. Rev. Genet.* 45:119-144.

Oono, R., and Denison, R. F. 2010. Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. *Plant Physiol.* 154:1541-1548.

Oono, R., Schmitt, I., Sprent, J. I., and Denison, R. F. 2010. Multiple evolutionary origins of legume traits leading to extreme rhizobial differentiation. *New Phytol.* 187:508-520.

Parra-Colmenares, A., and Kahn, M. L. 2005. Determination of nitrogen fixation effectiveness in selected *Medicago truncatula* isolates by measuring nitrogen isotope incorporation into pheophytin. *Plant Soil* 270:159-168.

Perret, X., Staehelin, C., and Broughton, W. J. 2000. Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* 64:180-201.

Radutoiu, S., Madsen, L. H., Madsen, E. B., Felle, H. H., Umehara, Y., Grönlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425:585-592.

Radutoiu, S., Madsen, L. H., Madsen, E. B., Jurkiewicz, A., Fukai, E., Quistgaard, E. M., Albrechtsen, A. S., James, E. K., Thirup, S., and Stougaard, J. 2007. LysM domains mediate lipochitin-oligosaccharide recognition and *Nfr* genes extend the symbiotic host range. *EMBO J.* 26: 3923-3935.

Rosenberg, C., Boistard, P., Dénarié, J., and Casse-Delbart, F. 1981. Genes controlling early and late functions in symbiosis are located on a megaplasmid in *Rhizobium meliloti*. *Mol. Gen. Genet.* 184:326-333.

Roux, B., Rodde, N., Jardinaud, M. F., Timmers, T., Sauviac, L., Cottret, L., Carrère, S., Sallet, E., Courcelle, E., Moreau, S., Debellé, F., Capela, D., de Carvalho-Niebel, F., Gouzy, J., Bruand, C., and Gamas, P. 2014. An integrated analysis of plant and bacterial gene expression in symbiotic root nodules using laser-capture microdissection coupled to RNA sequencing. *Plant J.* 77:817-837.

Snyman, C. P., and Strijdom, B. W. 1980. Symbiotic characteristics of lines and cultivars of *Medicago truncatula* inoculated with strains of *Rhizobium meliloti*. *Phytophactica* 12:173-176.

Sun, S., Guo, H., and Xu, J. 2006. Multiple gene genealogical analyses reveal both common and distinct population genetic patterns among replicons in the nitrogen-fixing bacterium *Sinorhizobium meliloti*. *Microbiology* 152:3245-3259.

Thoquet, P., Ghérandi, M., Journet, E. P., Kereszt, A., Ané, J. M., Prosperi, J. M., and Huguet, T. 2002. The molecular genetic linkage map of the model legume *Medicago truncatula*: an essential tool for comparative legume genomics and the isolation of agronomically important genes. *BMC Plant Biol.* 2:1.

Tirichine, L., de Billy, F., and Huguet, T. 2000. *Mtsym6*, a gene conditioning *Sinorhizobium* strain-specific nitrogen fixation in *Medicago truncatula*. *Plant Physiol.* 123:845-851.

Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., Farkas, A., Mikulass, K., Nagy, A., Tiricz, H., Satiat-Jeunemaitre, B., Alunni, B., Bourge, M., Kucho, K., Abe, M., Kereszt,

- A., Maroti, G., Uchiumi, T., Kondorosi, E., and Mergaert, P. 2010. Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science* 327:1122-1126.
- Wang, D., Griffiths, J., Starker, C., Fedorova, E., Limpens, E., Ivanov, S., Bisseling, T., and Long, S. 2010. A nodule-specific protein secretory pathway required for nitrogen-fixing symbiosis. *Science* 327: 1126-1129.
- Wang, D., Yang, S., Tang, F., and Zhu, H. 2012. Symbiosis specificity in the legume: rhizobial mutualism. *Cell. Microbiol.* 14:334-342.
- Wang, Q., Yang, S., Liu, J., Terecskei, K., Ábrahám, E., Gombár, A., Domonkos, A., Szűcs, A., Kőrmöcz, P., Wang, T., Fodor, L., Mao, L., Fei, Z., Kondorosi, É., Kaló, P., Kereszt, A., and Zhu, H. 2017. Host-secreted antimicrobial peptide enforces symbiotic selectivity in *Medicago truncatula*. *Proc. Natl. Acad. Sci. U.S.A.* 114:6854-6859.
- Xing, H. L., Dong, L., Wang, Z. P., Zhang, H. Y., Han, C. Y., Liu, B., Wang, X. C., and Chen, Q. J. 2014. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biol.* 14:327.
- Yang, S., Tang, F., Gao, M., Krishnan, H. B., and Zhu, H. 2010. *R* gene-controlled host specificity in the legume-rhizobia symbiosis. *Proc. Natl. Acad. Sci. U.S.A.* 107:18735-18740.
- Yang, S., Wang, Q., Fedorova, E., Liu, J., Qin, Q., Zheng, Q., Price, P. A., Pan, H., Wang, D., Griffiths, J. S., Bisseling, T., and Zhu, H. 2017. Microsymbiont discrimination mediated by a host-secreted peptide in *Medicago truncatula*. *Proc. Natl. Acad. Sci. U.S.A.* 114:6848-6853.
- Yasuda, M., Miwa, H., Masuda, S., Takebayashi, Y., Sakakibara, H., and Okazaki, S. 2016. Effector-triggered immunity determines host genotype-specific incompatibility in legume-Rhizobium symbiosis. *Plant Cell Physiol.* 57:1791-1800.