



# Host-secreted antimicrobial peptide enforces symbiotic selectivity in *Medicago truncatula*

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Contributed by Éva Kondorosi, February 14, 2017 (sent for review January 17, 2017; reviewed by Rebecca Dickstein and Julia Frugoli)

**Legumes engage in root nodule symbioses with nitrogen-fixing soil bacteria known as rhizobia. In nodule cells, bacteria are enclosed in membrane-bound vesicles called symbiosomes and differentiate into bacteroids that are capable of converting atmospheric nitrogen into ammonia. Bacteroid differentiation and prolonged intracellular survival are essential for development of functional nodules. However, in the *Medicago truncatula*–*Sinorhizobium meliloti* symbiosis, incompatibility between symbiotic partners frequently occurs, leading to the formation of infected nodules defective in nitrogen fixation (Fix<sup>-</sup>). Here, we report the identification and cloning of the *M. truncatula* *NFS2* gene that regulates this type of specificity pertaining to *S. meliloti* strain Rm41. We demonstrate that *NFS2* encodes a nodule-specific cysteine-rich (NCR) peptide that acts to promote bacterial lysis after differentiation. The negative role of *NFS2* in symbiosis is contingent on host genetic background and can be counteracted by other genes encoded by the host. This work extends the paradigm of NCR function to include the negative regulation of symbiotic persistence in host–strain interactions. Our data suggest that NCR peptides are host determinants of symbiotic specificity in *M. truncatula* and possibly in closely related legumes that form indeterminate nodules in which bacterial symbionts undergo terminal differentiation.**

legumes | rhizobial symbiosis | nitrogen fixation | symbiotic specificity | NCR peptides

**L**eguminous plants can provide their own nitrogen requirements by entering into a symbiosis with rhizobia, a diverse group of soil bacteria that have the ability to induce plants to form nitrogen-fixing root nodules. This symbiotic relationship is highly selective: Particular rhizobial species or strains establish an efficient symbiosis with only a limited set of legume species or genotypes (1, 2). Such specificity can occur at different stages of symbiotic development, ranging from initial nodule primordium induction and bacterial infection (nodulation specificity) to late nodule development involving bacterial differentiation and symbiotic persistence (nitrogen fixation specificity) (2). A comprehensive understanding of the genetic mechanisms that control this specificity has important implications in agriculture because it allows for genetic manipulation of the host or bacterial symbionts to optimize the agronomic potential of biological nitrogen fixation.

Establishing a nitrogen-fixing symbiosis requires the mutual recognition of a series of molecular signals between the symbiotic partners (3, 4). For this reason, multiple genetic and molecular mechanisms could be involved in the regulation of compatibility in the legume–rhizobial interactions (1, 2). In most legumes, nodule morphogenesis and bacterial infection is mediated by host-specific recognition of rhizobial lipo-chitooligosaccharides known as nodulation (Nod) factors. The Nod factors carry various species-specific chemical decorations, and this structural variation defines the basis of host–symbiont specificity, particularly at the species level (5, 6). In addition to Nod factors, rhizobia also use secreted

effectors or microbe-associated molecular patterns (MAMPs) such as surface polysaccharides to facilitate their invasion of the host (7, 8). Therefore, effector- or MAMP-triggered plant immunity mediated by intracellular nucleotide binding/leucine-rich repeat receptors or plasma membrane-localized pattern recognition receptors also plays an important role in determining host range of rhizobia (9, 10).

Development of nitrogen-fixing nodules involves simultaneous differentiation of both nodule and bacterial cells (11). During this process, intracellular membrane-bound bacteria become adjusted to the endosymbiotic lifestyle and develop into mature bacteroids capable of nitrogen fixation. Depending on the host, the morphology and physiology of bacteroids can be strikingly different (11). In galeoid legumes (e.g., alfalfa, peas, and clovers) that form indeterminate nodules, the bacteria often undergo terminal differentiation, which is characterized by cell enlargement coupled to genome amplification, increased membrane permeability, and loss of reproductive ability. In contrast, in the nongaleoid legumes (e.g., soybeans, common beans, and *Lotus japonicus*) that form determinate nodules, the bacteroids mostly retain the same morphology and can revert to the free-living form. Symbiotic specificity has also been documented at this phase of

## Significance

**Nitrogen is a limiting factor for plant growth. Most crops obtain their nitrogen through the use of nitrogen-based fertilizers, which is costly, and also causes environmental pollution. Legumes, however, have the unique ability to fix atmospheric nitrogen through symbioses with nitrogen-fixing bacteria. Although legumes can be nodulated by indigenous soil bacteria, nitrogen fixation efficiency differs significantly depending on host and bacterial genotypes. Understanding the genetic mechanisms that underlie this specificity will allow for optimizing symbiotic partnerships with improved symbiotic performance. We report that specific nodule-specific cysteine-rich (NCR) peptides negatively regulate symbiotic persistence in a strain-specific manner in *Medicago truncatula*. This finding offers a strategy to improve nitrogen fixation efficiency through selection or manipulation of NCR alleles that favor specific bacterial strains.**

Author contributions: Q.W., S.Y., É.K., P. Kaló, A.K., and H.Z. designed research; Q.W., S.Y., J.L., K.T., E.Á., A.G., Á.D., A.S., P. Körmöczi, T.W., and L.F. performed research; Z.F. contributed new reagents/analytic tools; Q.W., S.Y., J.L., K.T., E.Á., A.G., Á.D., A.S., P. Körmöczi, T.W., L.F., L.M., Z.F., É.K., P. Kaló, A.K., and H.Z. analyzed data; and É.K. and H.Z. wrote the paper.

Reviewers: R.D., University of North Texas; and J.F., Clemson University.

The authors declare no conflict of interest.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1700715114/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1700715114/-DCSupplemental).

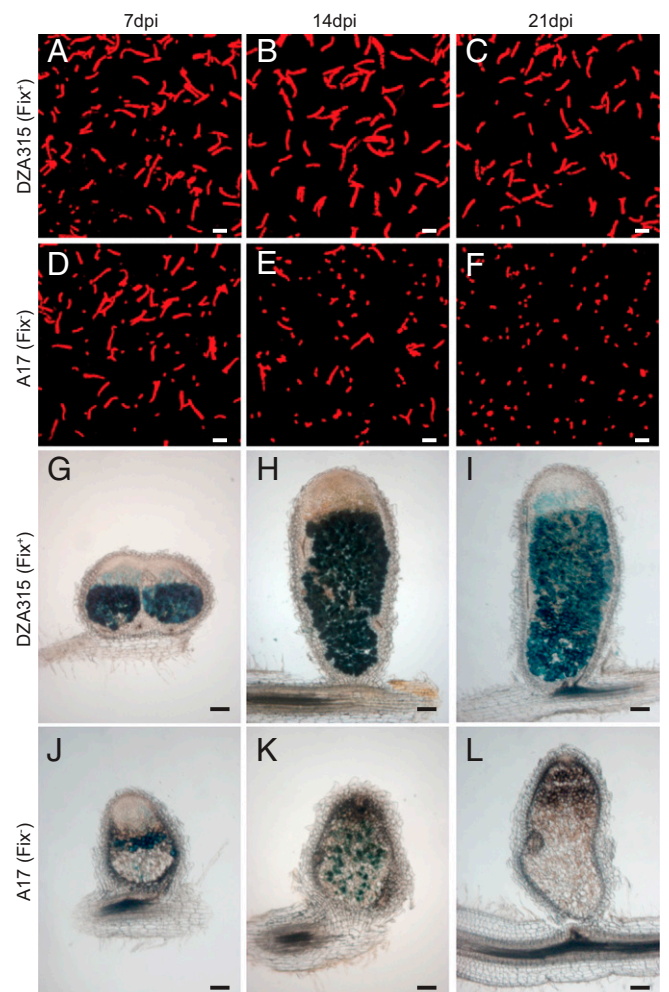
nodule development, which is mirrored by the presence of tremendous natural variation in nitrogen fixation efficiency between different plant–rhizobia combinations (12). In extreme cases, such specificity can lead to the formation of infected nodules that are unable to fix nitrogen, due to the failure of intracellular survival of the differentiated bacteroids (13–16). Despite recent advances in our understanding of the signaling pathways leading to initial recognition and nodule development (3, 4), host genetic control of this fixation-level incompatibility is not well understood.

*Medicago truncatula* establishes a symbiosis with its micro-symbioint *Sinorhizobium meliloti* (4). The symbiosis leads to the formation of indeterminate nodules, where the bacteria undergo terminal differentiation (11). The bacterial differentiation in *Medicago* nodules is associated with the expression of hundreds of nodule-specific cysteine-rich (NCR) host peptides (17–19). In this system, nitrogen fixation efficiency is dependent on genome-by-genome interactions between the symbiotic partners, and no single host genotypes or rhizobial strains are consistently associated with the best nitrogen fixation performance (12, 16). Frequently, the same bacterial strains can form either functional (Fix<sup>+</sup>) or nonfunctional (Fix<sup>−</sup>) nodules depending on the host genotype (14–16). Here, we report the isolation of the *M. truncatula NFS2* gene that is involved in regulation of the fixation-level incompatibility with *S. meliloti* strain Rm41. We show that *NFS2* encodes an NCR peptide that functions as a negative regulator of symbiotic persistence. Our work suggests that NCRs may be genetically manipulated to improve symbiotic nitrogen fixation in crop legumes such as alfalfa and peas.

## Results and Discussion

*S. meliloti* strain Rm41 forms Fix<sup>+</sup> nodules on the *M. truncatula* accession DZA315.16 (DZA315), but Fix<sup>−</sup> nodules on Jemalong A17 (A17) (15, 16). The Fix<sup>−</sup> phenotype of A17 results from its incompatibility with Rm41, because it can establish efficient symbioses with other strains (16). Rm41 bacteria are able to invade and colonize A17 nodule cells, but undergo lysis shortly after differentiation into elongated bacteroids (20) (Fig. 1 *A–F* and Fig. S1). Inoculation of plants by Rm41 carrying a β-glucuronidase (GUS) reporter gene driven by the *nifH* promoter showed that the *nifH* gene, encoding one component of the nitrogenase enzyme complex, was expressed in the young A17 nodules, further supporting the presence of differentiated bacteroids at early stages of nodule development; however, the expression was abolished 3 wk after inoculation due to bacterial lysis and nodule senescence (Fig. 1 *G–L* and Fig. S1). Accordingly, the expression of several early senescence marker genes was dramatically enhanced in A17 nodules compared with their expression levels in DZA315 nodules (Fig. S2).

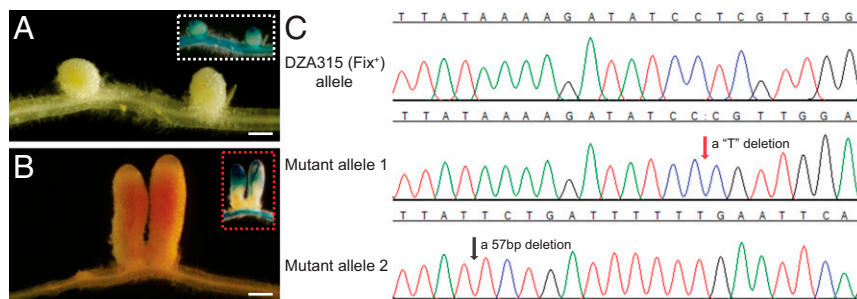
Genetic analysis in a recombinant inbred line (RIL) population derived from the cross of A17 and DZA315 suggested the involvement of multiple interacting loci in the control of this symbiotic specificity (20). From this RIL population, we identified a RIL that showed segregation of the Fix<sup>+</sup> and Fix<sup>−</sup> phenotypes. This segregation was caused by the residual heterozygosity around a locus termed *NFS2* (nitrogen fixation specificity 2), and we referred to this residual heterozygous line as RHL-*NFS2*. At this locus, the progeny homozygous for the DZA315 and A17 haplotypes formed Fix<sup>+</sup> and Fix<sup>−</sup> nodules, respectively. However, the heterozygotes displayed an intermediate phenotype (designated as Fix<sup>+/-</sup>) that can be reliably distinguished from the phenotypes of homozygotes (Fig. S3). Because the progeny of the residual heterozygous plants have a nearly identical homozygous genetic background, the genes that are polymorphic between the RNA pools of the Fix<sup>+</sup> and Fix<sup>−</sup> nodules are likely linked to *NFS2*. Using bulked segregant RNA-sequencing (RNA-seq) analysis (21), we mapped the *NFS2* locus to a small region on chromosome 8, which



**Fig. 1.** Differentiation and intracellular survival of the Rm41 bacteroids in the DZA315 (Fix<sup>+</sup>) and A17 (Fix<sup>−</sup>) nodules. (A–F) Confocal microscopy images of the bacterial populations isolated from the Fix<sup>+</sup> (A–C) and Fix<sup>−</sup> (D–F) nodules showing the gradual loss of elongated bacteroids and the accumulation of saprophytic bacteria in the Fix<sup>−</sup> nodules. (G–L) Expression of a GUS reporter gene driven by the *nifH* promoter in the Fix<sup>+</sup> (G–I) and Fix<sup>−</sup> (J–L) nodules showing that the *nifH* gene was expressed at the early developmental stages of the Fix<sup>−</sup> nodules, but its expression was abolished at later time points. dpi, days postinoculation. [Scale bars, 5 μm (A–F) and 200 μm (G–L).]

is linked to the *NFS1* locus described in the companion article (20). We designated the A17 allele of *NFS2* as *NFS2<sup>-</sup>* and the DZA315 allele as *NFS2<sup>+</sup>*. Fine mapping in the near-isogenic progeny of the residual heterozygous plants delimited the *NFS2* locus to a 120-kb genomic region (Fig. S4). This region encompasses, among other genes, *Medtr8g465280*, which encodes an NCR peptide (17). Because NCRs have been shown to be host effectors that mediate terminal bacteroid differentiation required for nitrogen fixation in the *Medicago*–*Sinorhizobium* symbiosis (18, 19, 22–24), we postulated that *Medtr8g465280* was a candidate gene of *NFS2*.

We tested the candidate gene in the near-isogenic background of RHL-*NFS2* through hairy root transformation. We first transformed the DZA315 (Fix<sup>+</sup>) allele of the candidate gene into the homozygous Fix<sup>−</sup> background (*NFS2<sup>-/-</sup>*) of RHL-*NFS2*. Contrary to our expectation, this experiment failed to complement the Fix<sup>−</sup> phenotype (Fig. 2*A*). Additionally, CRISPR/Cas9-mediated knockout of the DZA315 allele in the homozygous Fix<sup>+</sup> background (*NFS2<sup>+/+</sup>*) of RHL-*NFS2* retained the Fix<sup>+</sup> phenotype (Fig. 2*B* and C). Thus, we conclude that the DZA315



**Fig. 2.** Functional analysis of the DZA315 ( $\text{Fix}^+$ ) allele of the candidate gene *Medtr8g465280* in the RHL-NFS2 background. (A) Introduction of the DZA315 allele of *Medtr8g465280* into the  $\text{Fix}^-$  background of RHL-NFS2 failed to complement the  $\text{Fix}^-$  phenotype. (B) CRISPR/Cas9-mediated knockout of the DZA315 allele of *Medtr8g465280* in the  $\text{Fix}^+$  background of RHL-NFS2 retained the  $\text{Fix}^+$  phenotype. (Scale bars, 500  $\mu\text{m}$ .) (C) Sequence analysis identified two mutant alleles in the transgenic root forming  $\text{Fix}^+$  nodules shown in B. Insets show GUS staining of the same nodules/roots in the main images, as an indicator for the transgenic roots.

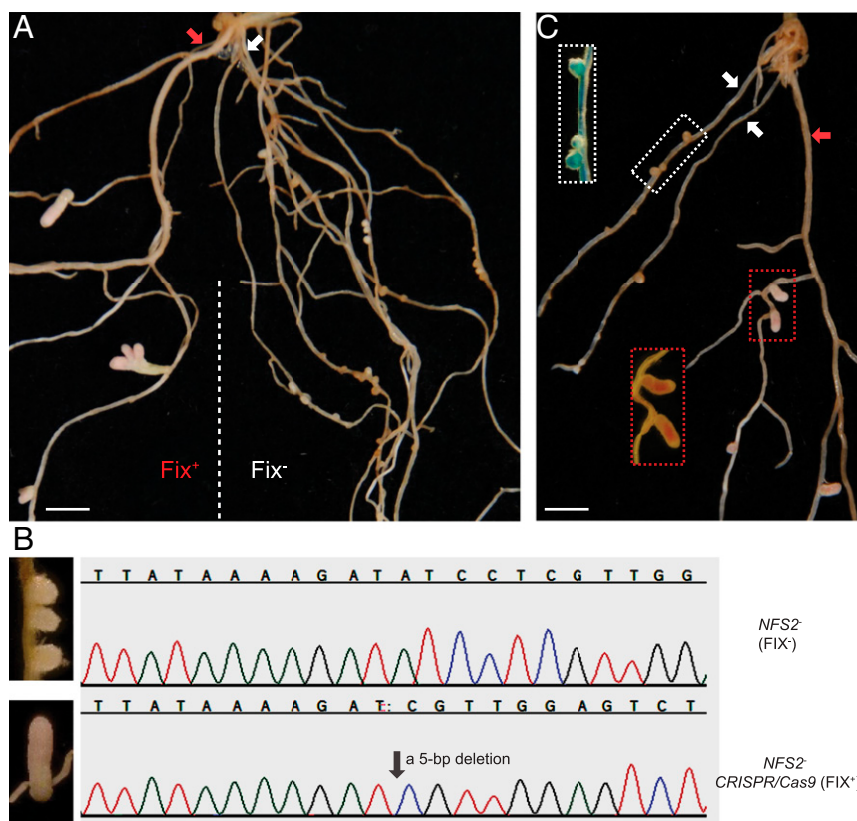
version of *Medtr8g465280* is not a functional allele required for forming  $\text{Fix}^+$  nodules by Rm41.

Because the heterozygotes displayed an intermediate phenotype, we asked the question of whether the A17 ( $\text{Fix}^-$ ) version of the candidate gene is a partially dominant (functional) allele that contributes to the development of the  $\text{Fix}^-$  phenotype. To address this possibility, we knocked out the A17 allele of *Medtr8g465280* in the *NFS2*<sup>-/-</sup> background of RHL-NFS2 using the CRISPR/Cas9 system. Intriguingly, knockout of the A17 allele converted the  $\text{Fix}^-$  phenotype to  $\text{Fix}^+$  (Fig. 3A and B). Moreover, expressing the A17 allele in the *NFS2*<sup>+/+</sup> background of RHL-NFS2 resulted in the formation of  $\text{Fix}^-$  nodules on the transgenic roots (Fig. 3C). Therefore, we conclude that the A17 version of *Medtr8g465280* is a functional allele that contributes to the development of  $\text{Fix}^-$  nodules by Rm41.

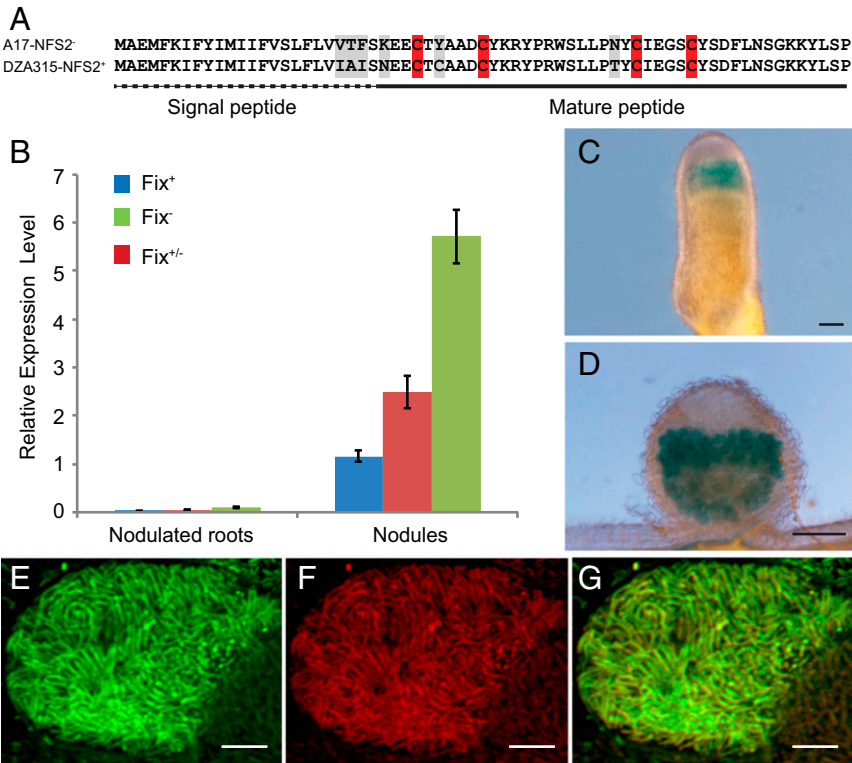
*NFS2* codes for a canonical NCR peptide of 67 amino acids (aa) (Fig. 4A). Cleavage of the predicted 24-aa signal sequence would produce a 43-aa mature peptide containing four conserved cysteine residues, with a predicted isoelectric point (pI) of 7.89 in A17. *NFS2*<sup>-</sup> (A17) and *NFS2*<sup>+</sup> (DZA315) mature peptides differ in three amino acid substitutions (Fig. 4A), leading to

a decrease of the predicted pI to 6.25 in DZA315. None of these substitutions occurred in the conserved cysteine positions. The expression of *NFS2*, like other *NCRs*, is restricted to root nodules, with *NFS2*<sup>-</sup> being expressed at a higher level than *NFS2*<sup>+</sup> (Fig. 4B). A promoter-GUS assay indicated that *NFS2* was predominantly expressed in cells around the transition between the infection and fixation zones in the  $\text{Fix}^+$  and  $\text{Fix}^-$  nodules (Fig. 4C and D), consistent with the expression pattern revealed by RNA-seq analysis of different nodule zones obtained with laser-capture microdissection (Fig. S5) (25). We also demonstrated that *NFS2*<sup>-</sup> peptide colocalized with bacteroids in the cells at the transition zone where the bacteria were still alive at 4 wk after inoculation (Fig. 4E-G).

NCRs resemble the cysteine-rich antimicrobial peptides in eukaryotes (26). This similarity is in line with the observations that some synthetic cationic peptides (pI > 9.0) showed strong bactericidal activities when applied to the free-living bacteria (19, 22, 23). Synthetic *NFS2*<sup>-</sup> peptide of A17, but not *NFS2*<sup>+</sup> peptide of DZA315, also possessed a mild bactericidal activity causing bacterial cell death of Rm41 in vitro, as evidenced by a reduction of colony-forming units in plating assays (Fig. 5A).



**Fig. 3.** Functional validation of the A17 allele of *Medtr8g465280* in the RHL-NFS2 background. (A) CRISPR/Cas9-mediated knockout of *Medtr8g465280* in the  $\text{Fix}^-$  background of RHL-NFS2 converted the  $\text{Fix}^-$  phenotype to  $\text{Fix}^+$ . The mutant root forming  $\text{Fix}^+$  nodules is indicated by the red arrow (left), and the wild-type roots forming  $\text{Fix}^-$  nodules is indicated by the white arrow (right). (Scale bar, 3 mm.) (B) Sequence analysis indicates a 5-bp deletion (indicated by an arrow) in the transgenic root-forming  $\text{Fix}^+$  nodules shown in A, whereas the roots forming  $\text{Fix}^-$  nodules are nontransgenic and contain the wild-type A17 allele. (C) Transgenic hairy roots expressing the A17 allele of *Medtr8g465280* in the  $\text{Fix}^+$  background of RHL-NFS2 led to the formation of  $\text{Fix}^-$  nodules (indicated by white arrows), whereas the nontransgenic nodules retained the  $\text{Fix}^+$  phenotype (marked by the red arrow). Insets show GUS staining of the same nodules/roots in the main image, as an indicator for the transgenic roots. (Scale bars, 3 mm.)



**Fig. 4.** Expression of *NFS2* and cellular localization of the *NFS2*<sup>-</sup> peptide. (A) Alignment of the *NFS2* peptides of A17 and DZA315. The amino acid substitution sites and conserved cysteine positions are highlighted. (B) The expression of *NFS2* is restricted to root nodules. (C and D) A promoter-GUS assay indicated that *NFS2*<sup>+</sup> was predominantly expressed around the transition of the infection and fixation zones in the *Fix*<sup>+</sup> nodules (C). In the *Fix*<sup>-</sup> nodules, the expression of *NFS2*<sup>-</sup> was spread to the proximal portion (D), but the nodule zones cannot be clearly defined. (Scale bars, 200  $\mu$ m.) (E–G) GFP-tagged bacteroids (E), *NFS2*<sup>-</sup>-mCherry (F), and an overlay (G) in a symbiotic cell from the transition zone shows that the *NFS2*<sup>-</sup> peptide colocalized with the symbiosomes before the bacteroids were killed. (Scale bars, 10  $\mu$ m.) The experiments were performed at 4 wk after inoculation.

Similar bacterial killing effect was also observed on *Sinorhizobium medicae* ABS7, a strain that establishes an efficient symbiosis with both A17 and DZA315 (Fig. 5B). This observation suggests that the in vitro antimicrobial activity of the peptide is not necessarily correlated with its in planta function (22, 23). The differential in vitro activity of the two peptides was also reflected by their effects on induction of the membrane permeability (Fig. 5C) and bacterial elongation (Fig. 5D–F). It remains to be determined how the amino acid substitutions affect the peptide activity.

Similar to *NFS1* described in the companion article (20), the negative effect of *NFS2*<sup>-</sup> on symbiotic persistence is dependent on host genetic background, because some *M. truncatula* accessions (Fig. S6) and RILs (Table S1) that have the *NFS2*<sup>-/-</sup> genotype can form *Fix*<sup>+</sup> nodules with *S. meliloti* Rm41. In particular, the *NFS2*<sup>-</sup> allele appears not to contribute to the development of *Fix*<sup>-</sup> nodules in the A17 genetic background, even though it plays a role in the RHL-*NFS2* genetic background, where the *NFS1*<sup>-</sup> function is suppressed. Indeed, knockout of *NFS1*<sup>-</sup> (but not *NFS2*<sup>-</sup>) is sufficient to allow Rm41 to fix nitrogen with A17 (Fig. 6), suggesting that the negative effect of *NFS2*<sup>-</sup> is counteracted by other factors in this background.

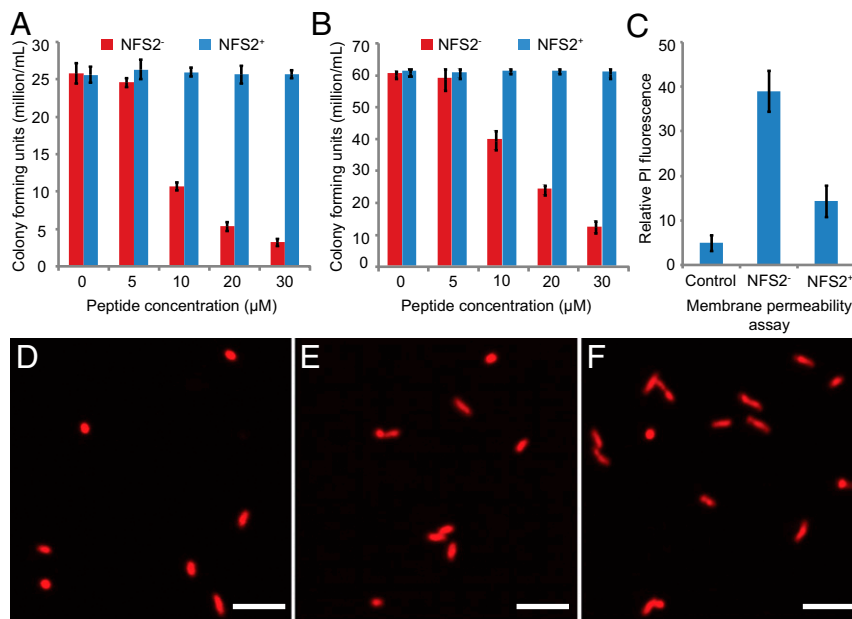
## Conclusion

*NCRs* are extremely abundant in *M. truncatula*, with >500 diverse family members (26). These genes are predominantly expressed in the infected nodule cells. Targeting these defensin-like peptides to symbiosomes is essential for terminal bacteroid differentiation required for nitrogen fixation (19, 27). It was speculated that the galeoid legumes such as *Medicago* have evolved *NCR* peptides to “enslave” rhizobial microsymbionts as terminally differentiated bacteroids with organelle-like properties optimized for nitrogen fixation (28–31). However, this study, together with the companion article (20), reveals that certain *NCRs* can negatively affect nitrogen fixation in a strain-specific manner. This finding may not be an exception, but suggests a common role of *NCRs* in controlling symbiotic specificity in *M. truncatula*; for example, the same genomic region is also responsible for symbiotic

incompatibility involving *S. meliloti* strain A145 (14). How can *NCR* peptides possess both prosymbiotic and antisymbiotic properties? These apparently opposing roles are reconciled when one considers that, if the “enslavement” process takes the rhizobia to the edge of their life to function at a symbiotic optimum, it should not be surprising that host and symbiont must be delicately tuned for ideal symbiotic performance. The success of this symbiotic partnership will also depend on the genetic composition of the invading bacteria; if the rhizobia cannot handle the antibacterial activity of the peptides, then they are selected away for that plant genotype. It follows from this complex interaction that many contrived host-strain pairings that are compatible at the level of nodulation may not be appropriately tuned for efficient nitrogen fixation. Our identification of *NCR* peptides as instruments of this evolutionary tuning may help explain their rapid amplification and diversification and makes them attractive agents for engineering legume–rhizobia pairs with improved agricultural properties.

## Materials and Methods

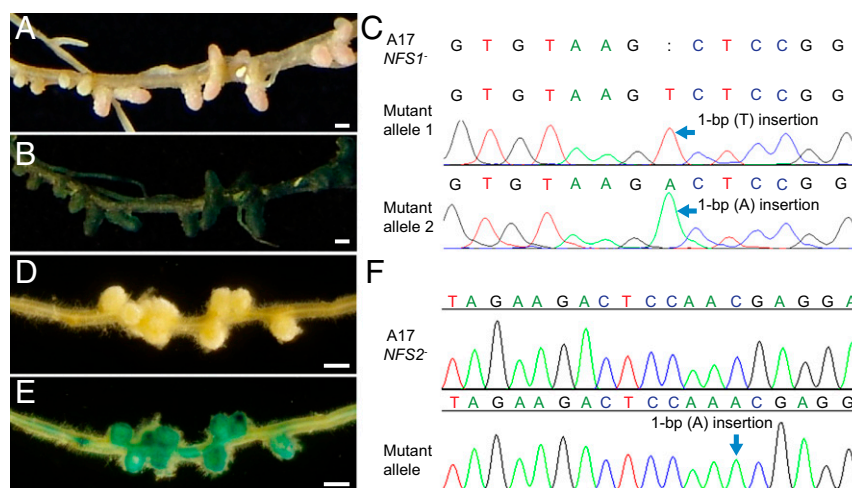
**Plant Materials, Nodulation Assay, and Genetic Mapping.** The *M. truncatula* seeds used in this study were originally provided by Jean-Marie Prosseri, Amélioration Génétique et Adaptation des Plantes Méditerranéennes et Tropicales, Institut National de la Recherche Agronomique, Montpellier, France. 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 analysis, roots of 1-wk-old seedlings were flood-inoculated with *S. meliloti* Rm41, and nodulation phenotypes were examined 3–4 wk after inoculation. Genetic mapping of the *NFS2* locus was based on the progeny of the residual heterozygous line RHL-*NFS2*. To rapidly identify the *NFS2* locus, RNA-seq was conducted on the two RNA pools derived from the *Fix*<sup>+</sup> and *Fix*<sup>-</sup> nodules. RNA-seq reads were first aligned to the *M. truncatula* reference genome (Version Mt4.0) (32, 33) by using the method described by Li and Durbin (34). After the alignment, SNPs and small indels (1–5 bp) were identified based on the mpileup files generated by SAMtools (35). The identified SNPs and small indels were supported by at least four reads and had an allele frequency of at least 0.8. Fine mapping of the *NFS2* locus was based on the SNPs identified between the two parental genotypes Jemalong A17 and DZA315. SNPs were genotyped either by converting to cleaved amplified polymorphic sequences markers or by direct sequencing.



**Fig. 5.** In vitro assay of the effects of synthetic NFS2 peptides on free-living bacteria. (A and B) Bactericidal assay of the peptides on *S. meliloti* Rm41 (A) and *S. medicae* ABS7 (B) showing that the NFS2<sup>-</sup> peptide significantly blocked the proliferation of both strains, whereas the NFS2<sup>+</sup> peptide had no effect on bacterial growth. Data are means  $\pm$  SD ( $n = 3$ ). (C) In vitro assay of the effects of the peptides on bacterial membrane integrity. PI uptake of *S. meliloti* Rm41 cells after treatment with NFS2<sup>-</sup> and NFS2<sup>+</sup> peptides showed that both peptides provoked membrane permeabilization, with NFS2<sup>-</sup> being more potent than NFS2<sup>+</sup>. The peptide concentration used was 30  $\mu$ M. Data are means  $\pm$  SD ( $n = 3$ ). (D–F) In vitro assay of the effects of NFS2 peptides on bacterial cell elongation. Control (D), NFS2<sup>+</sup>-treated (E), and NFS2<sup>-</sup>-treated (F) *S. meliloti* cells showing significant cell elongation upon treatment with the NFS2<sup>-</sup> peptide. Cells were stained with PI. [Scale bars, 5  $\mu$ m (D–F).]

**Plasmids and Vectors.** For complementation assays, the genomic DNA of the candidate gene was amplified and cloned into the binary vector pCambia1305.1 by using the In-Fusion Advantage PCR Cloning Kits (Clontech). The vector expressed a GUSPlus gene to facilitate the identification of transformed roots by GUS staining. The CRISPR/Cas9 gene knockout constructs were generated by using the pKSE401 vector described by Xing et al. (36). Two pairs of oligos were designed to target two different sites of *Medtr8g465280*. For site 1, we used the oligo pair 5'ATTGTGGGTAGAAGACTCCAACG3' and 5'AAACCGTTGGAGTCTTCTACCA3'; and for site 2, we used the oligos 5'ATTGTTA-TAAAAGATATCCTCGT3' and 5'AAACACGAGGATATCTTTTATAA3'. 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 Bsal-digested pKSE401 vector. We also amplified the GUSPlus gene cassette from pCambia1305.1 and ligated into the pKSE401 vector to facilitate the identification of transgenic roots. For promoter-GUS assay, a 1.8-kb fragment upstream of the translation start site of *Medtr8g465280* was first cloned into the pDONR/Zeo vector (Invitrogen) and then subcloned into the pMDC163 vector by using the Gateway cloning system (Invitrogen). The NFS2<sup>-</sup>-mCherry translational fusion construct was developed by in-frame fusion between the second exon of *NFS2*<sup>-</sup> and the mCherry coding sequence in pCambia1305.1. Genomic DNA of *NFS2*<sup>-</sup> was amplified by using the primer pair 5'-CCATGATTACGAATTCTGGACTTAGAGAGATTATCCGTT-3' and 5'-CGGGGACAAATATTTTTACT-3', and the mCherry sequence was amplified by primers



**Fig. 6.** Functional analysis of the *NFS1*<sup>-</sup> and *NFS2*<sup>-</sup> alleles in the A17 background. (A–C) Functional analysis of *NFS1*<sup>-</sup>. (A) CRISPR/Cas9-mediated knockout of *NFS1*<sup>-</sup> in the A17 background converted the Fix<sup>-</sup> phenotype to Fix<sup>+</sup>. (B) GUS staining of the same nodules/roots in A, as an indicator for the transgenic root. [Scale bars, 1 mm (A and B).] (C) Sequence analysis identified two mutant alleles in the transgenic root forming Fix<sup>+</sup> nodules shown in A and B. (D–F) Functional analysis of *NFS2*<sup>-</sup>. (D) CRISPR/Cas9-mediated knockout of *NFS2*<sup>-</sup> in the A17 background retained the Fix<sup>-</sup> phenotype. (E) GUS staining of the same nodules/roots in D, as an indicator for the transgenic root. [Scale bars, 1 mm (D and E).] (F) Sequence analysis identified a mutant allele in the transgenic root forming Fix<sup>-</sup> nodules shown in D and E.

5'-AAATATTGTCCTCCGATGGTGAGCAAGGGCAGGA-3' and 5'-CGAGTTCGACTCTAGACTACTGTACAGCTCGTCCA-3'.

**Hairy Root Transformation and Analysis of Transgenic Roots.** Hairy root transformations were based on the protocol described by Boisson-Dernier et al. (37) using *Agrobacterium rhizogenes* strain ARqua1. At least 100 plants were used for each transformation experiment, and the experiments were replicated at least two times. The transgenic roots were identified by GUS staining. For CRISPR/Cas9-based knockout experiments, we sequenced the candidate genes amplified from the transgenic roots to confirm the targeted mutations. In the case of the presence of multiple heterogeneous mutant alleles, the PCR products were cloned into the pGEM T-Easy vector (Promega), and at least 10 clones were sequenced.

**Microscopy.** For microscopic analysis, nodules were harvested at different time points after inoculation, fixed with 4% (wt/vol) formaldehyde in 1× PBS buffer (pH 7.4) for 30 min and then rinsed three times (15 min each) with 1× PBS buffer (pH 7.4). The nodules were embedded in 5% (wt/vol) agarose (SeaKem LE Agarose, Lonza), and 70- $\mu$ m-thick longitudinal sections were prepared with a Leica VT1200 Vibrotom (Leica Microsystems). To visualize the  $\beta$ -galactosidase and the rhizobial *nifH* promoter activity, nodule sections were stained and analyzed as described (23). To measure the length of rhizobia, bacterial populations were isolated at different time points after inoculation, stained with propidium iodide (PI), and photographed with an Olympus Fluoview FV1000 confocal laser scanning microscope. The length of all cells in 10 images of a certain time point was measured by the ImageJ software (Version 1.50i; <https://imagej.nih.gov/ij/index.html>).

**Analysis of Gene Expression.** RNAs were extracted by using the Plant RNeasy Mini Kit (Qiagen). Two micrograms of RNA were used for RT-PCR reactions using Moloney MLV reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed based on the instructions of the SsoAdvanced SYBR

Green Supermix Kit (Bio-Rad) on a CFX Connect Real-time System (Bio-Rad) by using gene-specific primers. The expression of *Mt-ubiquitin* was used for normalization. The PCR primers used for *NFS2* were 5'-TTTCCAAGGAAGAGTGATC-3' and 5'-TCAGAATAACAAGATCCTTCAATG-3', and for *Mt-ubiquitin*, they were 5'-GCAGATAGACACGCTGGGA-3' and 5'-CAGTCTTCAAACCTTTGGGCAG-3'. Three biological replications were performed.

**In Vitro Assay of Peptide Activities.** Synthetic NFS2 peptides were synthesized by LifeTein. The in vitro assay of peptide activities was performed based on the procedures described by Van de Velde et al. (19). Briefly, bacterial cultures were first grown to OD<sub>600</sub> of 0.3 in LB medium, washed with 5 mM Mes-KOH buffer at pH 5.8, and then diluted to OD<sub>600</sub> of 0.1 in the same buffer. A total of 200  $\mu$ L of bacteria were treated with the individual peptides at the indicated concentrations and incubated for 3 h at 30 °C. After the treatments, bacterial suspensions were serially diluted and plated out in triplicate on selective medium. Colony-forming units were counted after 2 d of incubation at 30 °C. For measuring membrane permeability, *S. meliloti* cells were diluted to OD<sub>600</sub> of 0.1 in minimal medium supplemented with 30  $\mu$ M individual peptides and incubated for 24 h at 30 °C. After incubation, PI was added to a final concentration of 10  $\mu$ g/mL. Cells were diluted to an appropriate concentration and visualized with an IX83 inverted microscope (Olympus).

**ACKNOWLEDGMENTS.** We thank T. Bisseling, E. Fedorova, D. Wang, and J. Griffiths for helpful comments on the manuscript. This work was supported by US Department of Agriculture/National Institute of Food and Agriculture Agricultural and Food Research Initiative Grant 2014-67013-21573 (to H.Z.); Kentucky Science and Engineering Foundation Grant 2615-RDE-015 (to H.Z.); Hungarian National Research Fund/National Research, Development and Innovation Office Grants 106068, 119652, and 120122/120300 (to P. Kaló, A.K., and Á.D.); and European Research Council Advanced Grant "Symbiotics" Grant 269067 (to É.K.). Confocal microscopy work was supported by National Science Foundation Cooperative Agreement 1355438.

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