An antimicrobial peptide essential for bacterial survival in the nitrogen-fixing symbiosis

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In the nitrogen-fixing symbiosis between legume hosts and rhizobia, the bacteria are engulfed by a plant cell membrane to become intracellular organelles. In the model legume *Medicago truncatula*, internalization and differentiation of *Sinorhizobium* (also known as *Ensifer*) *meliloti* is a prerequisite for nitrogen fixation. The host mechanisms that ensure the long-term survival of differentiating intracellular bacteria (bacteroids) in this unusual association are unclear. The *M. truncatula defective nitrogen fixation4* (*dnf4*) mutant is unable to form a productive symbiosis, even though late symbiotic marker genes are expressed in mutant nodules. We discovered that in the *dnf4* mutant, bacteroids can apparently differentiate, but they fail to persist within host cells in the process. We found that the *DNF4* gene encodes NCR211, a member of the family of nodule-specific cysteine-rich (NCR) peptides. The phenotype of *dnf4* suggests that NCR211 acts to promote the intracellular survival of differentiating bacteroids. The greatest expression of *DNF4* was observed in the nodule interzone II-III, where bacteroids undergo differentiation. A translational fusion of DNF4 with GFP localizes to the peribacteroid space, and synthetic NCR211 prevents free-living *S. meliloti* from forming colonies, in contrast to mock controls, suggesting that DNF4 may interact with bacteroids directly or indirectly for its function. Our findings indicate that a successful symbiosis requires host effectors that not only induce bacterial differentiation, but also that maintain intracellular bacteroids during the host–symbiont interaction. The discovery of NCR211 peptides that maintain bacterial survival inside host cells has important implications for improving legume crops.

nitrogen-fixing symbiosis | *Sinorhizobium meliloti* | *Medicago truncatula* | legume | NCR antimicrobial peptides

Many legume plants satisfy their nitrogen needs by interacting with nitrogen-fixing bacteria (rhizobia) to form a specialized symbiotic organ, the root nodule. As rhizobia penetrate root hair cells through invaginations of host membrane called infection threads, the cortical cells underneath start dividing and eventually build the nodule in which the invading rhizobia are internalized to form intracellular organelles known as symbiosomes.

Among legumes, *Medicago truncatula* belongs to a group that forms indeterminate nodules, where a meristem continuously produces new nodule cells. An indeterminate nodule can be divided into four zones harboring different cell types (1). The meristem of indeterminate nodules is located in the apical region (zone I), which constantly supplies new cells to the nodule. These new cells then become infected with rhizobia in zone II, where the rhizobial cells are released from the infection thread into the host cytoplasm to form symbiosomes. On release into the host cytoplasm, the bacteroids are encapsulated with a host-derived membrane (the peribacteroid membrane), blocking the rhizobia from directly contacting the cytoplasm. The released bacteroids multiply and gradually colonize the host cell. In interzone II-III, rhizobial *nif* genes are turned on as the bacteroids expand, primarily by elongation, occupying a majority (~65%) of the host cell volume. The volume of vacuoles decreases dramatically, down to ~30% of the cell volume, which is correlated with the suppression of HOPS (homotypic fusion and vacuole protein sorting complex) gene expression in infected cells (2). In zone III, as the infected cells stop expanding, bacteroids become terminally differentiated and actively convert atmospheric nitrogen into ammonia, which can be readily transferred to the host plant for assimilation into amino acids. In older nodules, zone IV (also called the senescence zone) is present, where the host cells and bacteroids degenerate.

Although in certain legumes the nitrogen-fixing bacteroids remain morphologically similar to free-living bacteria and are capable of reverting back to the nonsymbiotic lifestyle, bacteroids in nodules formed on the inverted-repeat lacking clade (IRLC) of legumes, such as *M. truncatula*, undergo remarkable transformations to differentiate terminally, such that they are no longer able to survive independent of their host. Features of bacterial differentiation include genome amplification and cell elongation, and the host cell also expands by endoreduplicating its own genome (3, 4). Studies have shown that the differentiation of bacteroids requires the delivery of host effectors, such as nodule-specific cysteine-rich (NCR) peptides, through the endoplasmic reticulum secretory system (5, 6). Present only in IRLC legumes, NCRs are defensin-like antimicrobial peptides, some of which drive the differentiation of bacteroids (5, 7, 8). They are now recognized as major players in the interaction between the legume host and rhizobia. The importance of this family is underscored by their massive expansion in the IRLC lineage, with more than 500 members encoded in the *M. truncatula* genome.

The best evidence for the requirement of the NCR peptides to date has been obtained by disrupting the nodule-specific protein secretory pathway, where intracellular rhizobia no longer differentiate (6). However, blocking protein secretion in the nodule

Significance

Legumes form a root structure, the nodule, in which nitrogen-fixing bacteria (rhizobia) reside. In this symbiotic relationship, the bacteria provide nitrogen to the plant and in return obtain fixed carbon from the host. Once released into the cytoplasm of the host cell, the rhizobia undergo a remarkable transformation, including genome amplification and cell elongation, before reaching the differentiated nitrogen-fixing state. Small plant-derived peptides with antimicrobial activities have been known to play critical roles in the differentiation of rhizobia in legumes that form indeterminate nodules. By studying the *Medicago truncatula dnf4* mutant, we discovered that an antimicrobial peptide, NCR211, plays a critical role in the survival and function of differentiated rhizobia in host cells for successful symbiotic nitrogen fixation.

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indiscriminately is a blunt instrument that offers no insight into the specificity of individual NCR peptides. The large size of the NCR family and the limited sequence homology among its members hinder efforts to generalize their role in the symbiosis. Their sequence diversity and distinct temporal and spatial expression patterns have led to the suggestion that they may perform diverse functions, with different bacterial targets and modes of action (9), but direct proof of functional diversity is still lacking.

Our best understanding is of the subset of peptides that induce bacterial differentiation. NCR247 has been studied in particular detail, and has been reported to enter bacteroids and interact with a plethora of proteins (10). In addition, NCR247 treatment causes massive transcriptome changes in the bacteria (~15% of the *Sinorhizobium meliloti* genome), affecting critical cell cycle regulators and cell division genes (11). Genetically, the only in planta evidence supporting the role of NCR peptides in bacterial differentiation comes from ectopic expression of NCR035 in *Lotus japonicus* (5). Although loss-of-function genetic results would be desirable, it is generally assumed that within such a large group, the contribution from any single NCR peptide will not be sufficient for its absence to cause a significant phenotype.

Here, by studying the *defective in nitrogen fixation* mutant, *dnf4*, we report that an individual NCR peptide can be indispensable for the nitrogen-fixing symbiosis. The symbiotic mutant phenotype indicates that the *M. truncatula DNF4* gene is required for the survival and function of differentiating bacteroids. Map-based cloning followed by whole genome sequencing identified the *DNF4* gene as encoding NCR211. Our results indicate that while some NCRs such as NCR247 have been suggested to induce bacterial differentiation, DNF4/NCR211 on the other hand acts on protecting differentiating bacteroids in symbiosomes from degeneration.

Results

***dnf4* Is Defective in Maintaining Differentiating Bacteroids Inside the Host Cell.** The *dnf4* mutant was isolated from a screen for *M. truncatula* mutants defective in nitrogen fixation (12). In growth medium lacking exogenous nitrogen, the inability of *dnf4* mutant plants to use atmospheric nitrogen resulted in growth retardation and chlorotic leaves (Fig. 1*A*). The nodules of *dnf4* also remained small and white, whereas wild type nodules elongated and developed a pink color, which derives from the accumulation of leghemoglobin (Fig. 1*B*). Toluidine blue-stained nodules of *dnf4* at 10 d postinoculation (dpi) appeared normal in zones I and II, as well as in interzone II-III, where the dark color suggests that the host cells were fully occupied by bacteria (Fig. 1*C*); however, in the nitrogen-fixing zone III, bacteroids appeared to disappear from the central part of some host cells proximal to the root. Under higher magnification, fully infected *dnf4* cells exhibited a morphology similar to that of WT cells, with elongated bacteroids arranged around the central vacuole; however, cells that may have once contained bacteroids were clearly degenerated (Fig. 1*D*).

To further analyze the nature of the defect in *dnf4*, we examined the activity of the *nifH* bacterial gene using a promoter fusion construct with the *GUS* gene that encodes β-glucuronidase (12). The *nifH* gene encodes one of the subunits of the nitrogenase enzyme complex, and thus is required for nitrogen fixation. At 10 dpi, nodules of wild type and *dnf4* plants showed similar *nifH* expression (Fig. 2*A*); however, in *dnf4* nodules, *nifH* expression was lost by 14 dpi (Fig. 2*B*), whereas younger nodules that formed later on the lateral roots (arrowhead in Fig. 2*B*) still displayed *nifH* expression. These data indicate that the *nifH* gene was expressed in young *dnf4* nodules, but its expression was abolished in older ones.

Host leghemoglobin proteins bind free oxygen to create a microoxic environment in nitrogen-fixing cells, protecting the nitrogenase enzyme from inactivation by oxygen. *nifH* is expressed only in low-oxygen conditions, implying that leghemoglobin is

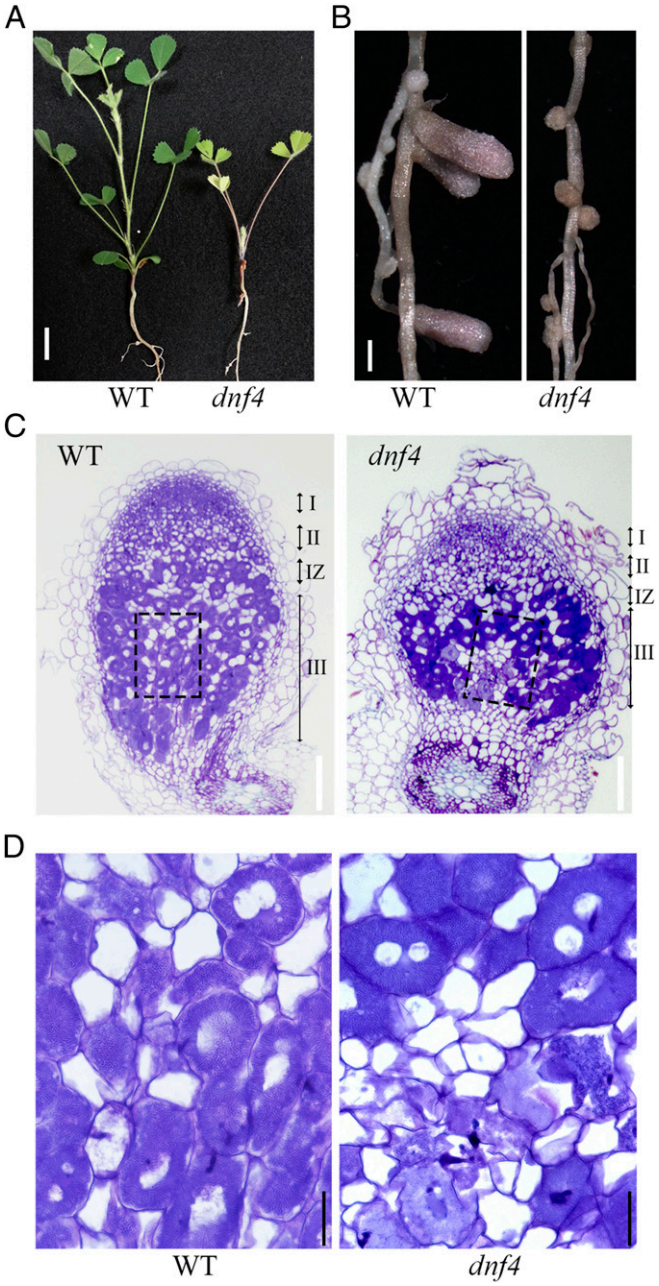

Fig. 1. *dnf4* is unable to develop functional nodules. (*A*) Plants were inoculated with *S. medicae* ABS7 and grew for 4 wk. Yellow leaves are the sign of nitrogen deficiency. (Scale bar: 10 mm.) (*B*) *dnf4* nodules remain small and white, whereas the wild type nodules elongate and show pink color. (Scale bar: 1 mm.) (*C*) Toluidine blue staining of 10-dpi nodules inoculated with ABS7. (Scale bar: 100 μm.) (*D*) Magnification of boxed areas in *C*. Elongated bacteroids stain dark blue. *dnf4* nodules show degradation of symbiosomes and host cells at the nitrogen fixation zone proximal to the root. (Scale bar: 20 μm.)

present in *dnf4* nodules. We observed that 10–20% of *dnf4* nodules were slightly pink, suggesting that these nodules express leghemoglobin proteins. This proposition was confirmed by immunoblot analyses of proteins extracted from 10-dpi nodules. The 10-dpi nodules of *dnf4* also accumulated leghemoglobin proteins at a level near that measured in wild type, in contrast to the early nodulation mutant *dnf1*, in which leghemoglobin protein was not detected (12) (Fig. 2*C*). Thus, *dnf4* nodules are deficient in the symbiosis even though both partners express genes characteristic

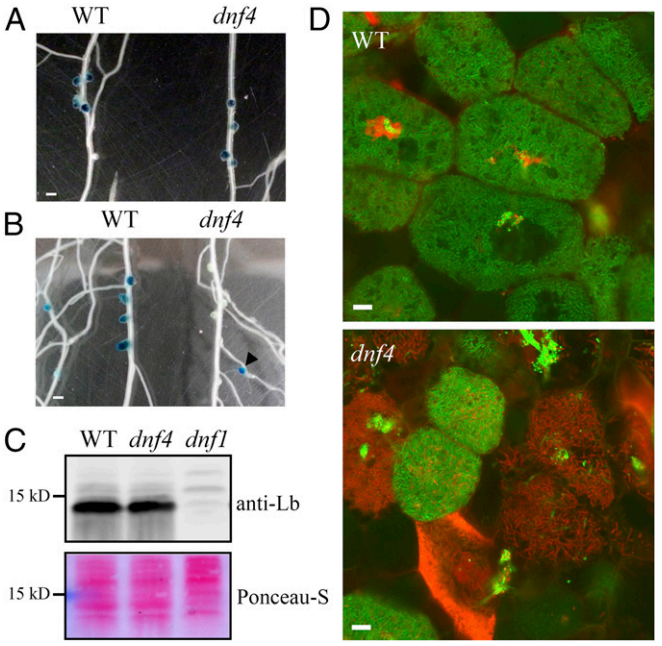

Fig. 2. *dnf4* nodules express fixation genes but fail to sustain differentiated bacteroids. (*A* and *B*) Activity of a *nifH::GUS* reporter in WT and *dnf4* nodules at 10 dpi (*A*) and 14 dpi (*B*). The arrowhead in *B* denotes a young *dnf4* nodule on a lateral root. (Scale bar: 1 mm.) (*C*) Accumulation of leghemoglobin in 10-dpi nodules. Ponceau S-stained membrane is shown as a loading control. (*D*) Live/dead staining of bacteroids in *dnf4* compared with WT nodules (14 dpi). Live bacteroids have a green signal (GFP) and dying bacteroids have a red signal from propidium iodide (PI). PI can only enter bacteroids with compromised membranes. (Scale bar: 10 μm.)

of mature nitrogen-fixing nodules. These results are also consistent with a previous report of the *dnf4* mutant being defective in later stages of nodule development, unlike mutants affected in earlier stages of nodule development, such as *dnf1*, *dnf2*, and *dnf5* (12).

To observe microscopic details of *dnf4* defects, mutant nodules were inoculated with GFP-expressing *S. meliloti* Rm1021 and stained with propidium iodide (PI), a fluorescence dye that can penetrate only compromised biological membranes. Wild type nodules at 14 dpi had few PI-positive bacteroids. In contrast, *dnf4* nodule cells showed widespread PI staining in bacteroids. Furthermore, *dnf4* bacteroids, including ones staining positive for PI, appeared elongated and differentiated (Fig. 2*D*), unlike other early-stage mutants (6, 13), although the degree of bacteroid differentiation was unclear. Coupling PI staining with SYTO9, a commonly used viability dye, yielded a similar pattern. In WT nodules, most nitrogen-fixing bacteroids were green (Fig. S1*A*). In *dnf4*, most cells containing differentiated bacteroids were red, suggesting that the bacteria in these cells were dead; however, bacteria in recently infected cells were still alive, and in some nodules, these newly infected cells formed a sharp band in the infection zone, suggesting that the infection process itself was normal (Fig. S1*B*). Some bacteroids in *dnf4* cells appeared to reach the final stage of differentiation, when they are radially aligned (Fig. S1 *C* and *D*). In summary, the phenotypic characterization of *dnf4* nodules strongly suggests that this mutation causes defects in the maintenance of differentiating bacteroids within host cells.

Deletion of *NCR211* Is Responsible for the *dnf4* Phenotype. To identify the causal mutation of the *dnf4* phenotype, we crossed the *dnf4* mutant (in the Jemalong background) with the A20 ecotype. Using map-based cloning, we narrowed the region to the upper arm of chromosome 4, between molecular markers 003F07 and h2_133k2b. Genomic DNA pooled from phenotypically mutant plants was sequenced. An unrelated line in the A17 background (derived from Jemalong) was also sequenced as a reference. Alignment of sequence reads to the A17 reference genome identified a deletion of ~35 kb unique to *dnf4* close to the mapping interval (Fig. 3*A*). This deletion, verified by PCR amplification (Fig. S2), eliminated five predicted genes. Two of these five genes encode NCR211 and NCR178, both of which are expressed in nodules, whereas the other three genes encode hypothetical proteins not expressed in nodules according to available RNA-seq data (www.jcvi.org/medicago/index.php).

The two *NCR* genes were introduced into the *dnf4* mutant by hairy root transformation to test their ability to rescue the *dnf4* phenotype. Only the NCR211-encoding gene (Medtr4g035705) proved capable of complementing the *dnf4* mutant, confirming that it corresponds to *DNF4* (Fig. 3*B*). The rescue of *dnf4* phenotype by *NCR211* was observed in all transgenic plants ($n \geq 10$) selected by the red fluorescence of the DsRED1 marker gene.

The *DNF4* gene is predicted to produce a 58-aa-long preprotein. Cleavage of the signal peptide [the first 24 residues, as predicted by SignalP (14)] would yield an anionic (pI = 5.38) NCR211 peptide 34 aa long, with four conserved cysteines. A BLASTp search identified NCR178 (Medtr4g035725) as the closest homolog of the mature DNF4 (61.8% identity), suggesting that these two *NCR* genes may have arisen from a recent local duplication event. Our phenotypic analysis of the *dnf4* mutant suggests that NCR211's function is necessary to maintain the viability of differentiating bacteroids.

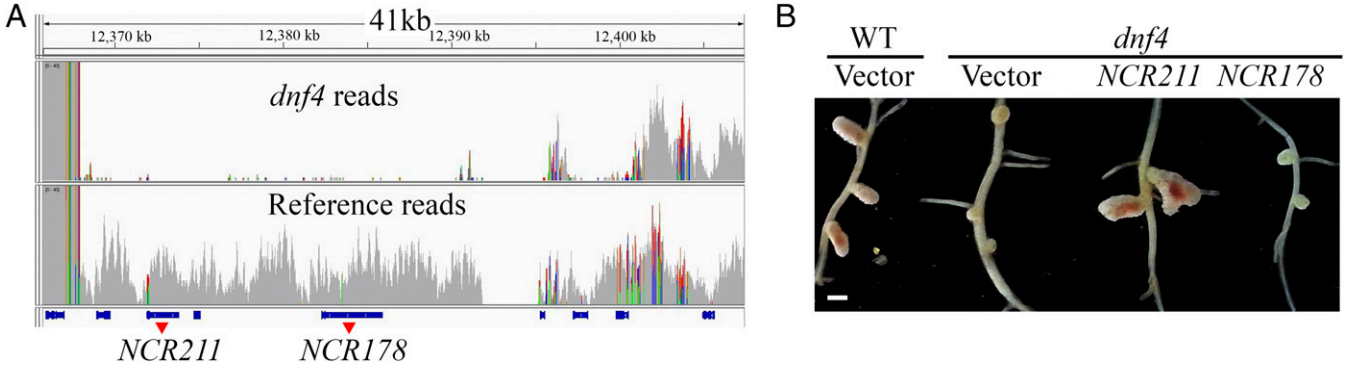

Fig. 3. Identification of the *DNF4* gene. (*A*) Read density plot of *dnf4* mutant (pool of mapping population) and an unrelated EMS mutant as a reference, showing that the deletion is unique to *dnf4*. The blue bars on the bottom represent gene models. (*B*) Nodule morphology on hairy roots transformed with empty vector or *NCR211* or *NCR178* genomic constructs. (Scale bar: 1 mm.)

***dnf4* Is Allelic to *esn1*.** We noticed that the defects in *dnf4* are similar to two *M. truncatula* symbiotic mutants deficient in nitrogen fixation, FN6753 and FN9669 (named *early senescent nodule1*, or *esn1*) (15) from a different irradiation-generated mutant collection. Under nitrogen-replete conditions, FN6753 and FN9669 are indistinguishable from wild type (15). Inoculated with *S. meliloti* Rm1021 under nitrogen depletion, FN6753 and FN9669 exhibited symbiotic phenotypes resembling the phenotype of the *dnf4* mutant (Fig. S3 *A*–*C*) (15). To assess whether the symbiotic defects of FN6753 and FN9669 result from mutations in *NCR211*, we first examined whether *NCR211* is deleted in these mutants. PCR results showed that *NCR211* is absent in both mutants (Fig. S3*D*). We next carried out a linkage analysis using a backcrossed F2 population of the *esn1* mutant. Wild type and *esn1* mutants segregated at a ratio close to 3:1 (95 wild type and 44 mutant), and the deletion in *NCR211* was linked to the symbiotic phenotype (Fig. S3*E*). Based on these results, we conclude that FN9669 and FN6753 represent additional alleles of the *dnf4* mutant.

***DNF4* Is Highly Expressed in Nodules and Localizes to the Symbiosome.** Microarray analysis of *NCR* genes during nodule development allowed us to search for the expression pattern of both *DNF4* (*NCR211*) and *NCR178* on Rm1021 inoculation (16). As shown in Fig. 4*A*, both genes were induced starting at 4 dpi, and their expression level continued to increase up to 40 dpi; however, the expression was much higher (by ~22-fold at 14 dpi) for *DNF4* compared with *NCR178*. Spatial gene expression profiling of Sm2011-inoculated nodules was recently performed with laser-capture microdissection (LCM) followed by RNA-seq (17). Data from this study indicate that expression of *DNF4* was high in the proximal half of zone II and reached its highest level in interzone II-III (Fig. 4*B*). *NCR178* exhibited a similar expression pattern (Fig. S4*A*). To confirm this pattern derived from LCM, promoter-*GUS* constructs of *DNF4* and *NCR178* were introduced into *M. truncatula* with hairy root transformation. Consistent with the LCM data, the promoter activity of *DNF4* was highest in interzone II-III (Fig. 4*C*). GUS activity was also present in zone III, as well as in proximal zone II. The promoter of *NCR178* showed an expression pattern similar to that of *DNF4* (Fig. S4*B*). The promoter-*GUS* expression analyses were performed with nodules from more than five independent transgenic roots, with similar results.

Previous studies of some NCR peptides showed localization inside bacteroids or on the bacteroid membrane (5, 10). Here the genomic sequences of *DNF4* and *NCR178* were fused in frame with a *GFP* sequence to create translational fusion constructs for localization studies. The constructs were introduced into both wild type and *dnf4* plants by hairy root transformation. The *DNF4-GFP* construct was able to fully rescue the defective nodule phenotype of *dnf4* in all transgenic plants recovered (n >10), indicating that the GFP fusion does not interfere with the function of DNF4 (Fig. S4*C*). DNF4-GFP was observed in the peribacteroid space between the host and the bacteroid membranes (Fig. 4*D*). An NCR178-GFP fusion protein (in wild type background) also localized to the peribacteroid space (Fig. S4*D*). The localization of DNF4 to the symbiosome suggests that the NCR211 peptide may directly or indirectly interact with bacteroids.

Exogenously Supplied NCR211 Does Not Reverse Membrane Permeability of the Symbiosome. The mature DNF4 peptide has certain sequence similarities with the scorpion toxin BmKK2, which acts as a blocker of eukaryotic potassium channels embedded in the cell membrane (18). As the DNF4 peptide localizes to the symbiosome, it could be in close proximity to three membranes: the host peribacteroid membrane, the bacterial outer membrane, and the bacterial inner membrane. Bacteroids in *dnf4* nodules readily take up PI, suggesting that all three membranes are permeabilized. To test whether DNF4 acts on the membranes of differentiated symbiosomes to block membrane permeabilization, we applied a synthetic NCR211 peptide to *dnf4* symbiosomes either extracted from nodules or in situ, and then proceeded with PI staining. In neither case did NCR211 prevent the uptake of the PI dye (Fig. S5). Along with later tests demonstrating that the synthetic NCR211 peptide is biologically active (next section and Fig. 5), these results suggest that NCR211 might not block membrane permeability in the symbiosome.

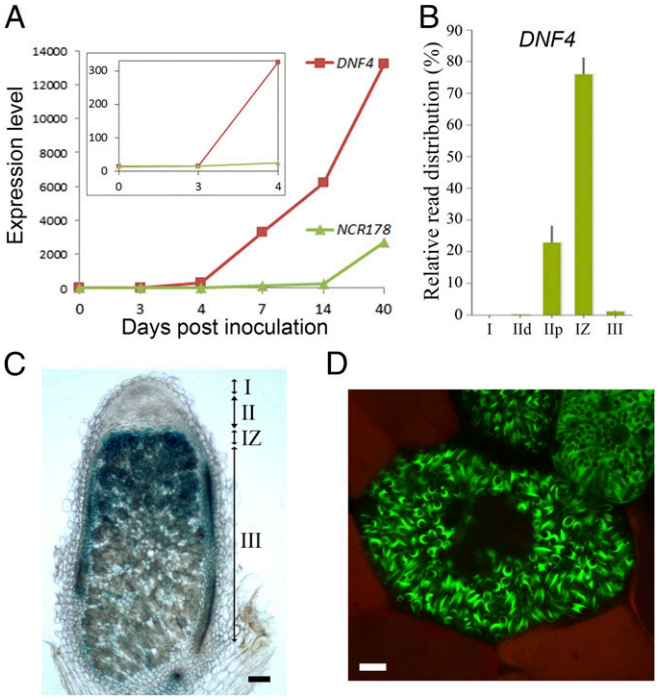

Fig. 4. *DNF4 (NCR211)* is highly expressed in interzone II-III, and the GFP fusion protein localizes to the peribacteroid space. (*A*) Temporal expression pattern of *DNF4* and *NCR178* after inoculation with Rm1021. (*Inset*) Expression at early time points. (*B*) Spatial expression pattern of *DNF4*. I, zone I; IId, zone II distal; IIp, zone II proximal; IZ, interzone II-III; III, zone III. (*C*) *DNF4* promoter GUS activity. (Scale bar: 100 μm.) (*D*) Localization of DNF4-GFP translational fusion in the peribacteroid space. (Scale bar: 10 μm.)

DNF4 Blocks the Proliferation of Free-Living Rhizobia. Although the synthetic NCR211 peptide does not appear to repair compromised symbiosome membranes, the localization pattern of DNF4 nonetheless suggests that it may interact with the bacteroids in some fashion. To test this hypothesis, we applied synthetic NCR211 and a different NCR peptide, NCR247, to free-living *S. meliloti* Rm1021. NCR247 treatments blocked subsequent colony formation, as reported previously (5). NCR211 treatments also blocked colony formation (Fig. 5). Although the kinetics of the two NCR peptides appeared different, at the highest concentration the inhibitory effect of NCR211 was comparable to that of NCR247 (Fig. 5).

Discussion

Approximately 600 genes encoding NCR peptides are predicted in the *M. truncatula* genome (19). The lack of symbiotic mutants in this gene family to date has been attributed to gene redundancy (16). Here we report that removing a single NCR peptide (NCR211) leads to defects in nitrogen fixation. Phenotypic characterization suggests that *dnf4* causes problems in the symbiosis at stages later than other *dnf* mutants, such as *dnf1*, *dnf2*, and *dnf5*, because a number of late symbiotic genes from bacteria (*nodF*, *bacA* and *nifH*) and plants (*LB1*, *CAM1* and *Nodulin* 31) are still expressed in *dnf4* (12). This classification is supported by our microscopic analyses demonstrating that differentiating bacteroids are present in *dnf4* mutant nodules, in contrast to those of *dnf1*

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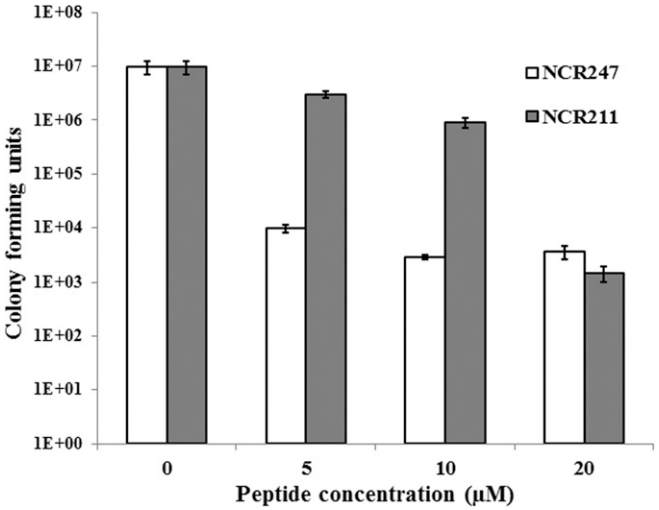

Fig. 5. A synthetic NCR211 peptide blocks the proliferation of free-living *S. meliloti* Rm1021. Bacteria were treated with either NCR211 or NCR247 for 3 h at the indicated concentrations, and spotted on solid media. The number of colonies was recorded 2 d later. Error bars indicate SDs ($n = 3$). The graph represents a typical outcome from multiple (>3) replications.

and *dnf2*, in which the bacteroids barely differentiate (6, 13). Furthermore, PI staining of differentiated bacteroids in the *dnf4* mutant suggests that NCR211 functions to maintain the viability of differentiating bacteroids. The loss of bacterial viability in *dnf4* is further supported by the lack of signals from the bacterial GFP reporter in PI-stained bacteroids (Fig. 1*D*), because doubly fluorescent bacteroids are rarely observed.

The deletion in the *dnf4* mutant includes two *NCR* genes, *NCR211* and *NCR178*. Although these are the closest homologs based on mature peptide sequences, only *NCR211* can rescue the *dnf4* phenotype. The promoter-GUS activity, LCM RNA-seq, and microarray data show similar temporal and spatial expression patterns for *NCR211* and *NCR178*; however, the expression level of *NCR211* is much higher than that of *NCR178*. Therefore, the lack of complementation of *dnf4* by *NCR178* might be related to its lower transcript level. An alternative explanation is that the differences in sequences of the two peptides might be sufficient to confer different biochemical activities and distinct biological functions. Promoter swapping analysis of the two genes could answer this question.

Structurally similar to defensins, some NCR peptides have bactericidal activities at high concentrations. At lower concentrations, certain NCR peptides induce membrane permeabilization, genome amplification, and cell elongation, which are features of terminal bacteroid differentiation in IRLC legumes (5). One of the NCR peptides, NCR247, was recently shown to bind many bacterial proteins, including ribosomal proteins and GroEL, indicating that this peptide may control bacteroids through multiple mechanisms (10). Massive transcriptome changes (~15% of the genome) by NCR247 are likely the result of numerous compromised cellular targets (10).

DNF4 is very different from NCR247 in terms of amino acid composition, charge, and length. Furthermore, whereas NCR247 has been reported to penetrate bacteroid membranes, DNF4-GFP is observed in the peribacteroid space (Fig. 4*D*), although this observation requires further verification using additional methods. The fact that nodules without *DNF4* contain differentiating bacteroids indicates that DNF4 acts after the bacteroids have initiated the differentiation program. Based on these observations, we propose that DNF4 functions to promote the survival of intracellular bacteroids during their differentiation. The expression of *DNF4* is high in the older parts of zone II and the highest in interzone II-III, where bacteroids undergo the final steps of differentiation before reaching maximum size. It appears that *DNF4* may be maximally expressed in these cells to protect terminally differentiating bacteria from otherwise lethal conditions. In the absence of DNF4, these bacteria perish either before or after reaching full differentiation. The expression pattern of *DNF4* is similar to that of *NCR247* and also overlaps with the pattern of *NCR035* in interzone II-III (5, 10); therefore, DNF4 could be in a position to balance the differentiation induced by other NCRs, such as NCR247 and NCR035. Finally, our finding that synthetic NCR211 is active on *S. meliloti* in culture suggests that this effect may be direct.

At first sight, it may appear perplexing that DNF4/NCR211 supports the survival of differentiating bacteroids in planta while also blocking free-living bacteria from forming colonies in culture. These two activities may reflect the same mode of action by NCR211 on bacterial biology, however. Differentiating bacteroids and free-living bacteria have different physiologies. The manipulation of the same bacterial target by DNF4 in these two very different physiological contexts could manifest itself in distinct outcomes. For bacteria, differentiation can be stressful, or even lethal if left unprotected. The induction of *DNF4* could provide a timely intervention to establish a sublethal, stable state in bacteroids. DNF4's action may be detrimental to free-living and proliferating bacteria, however. Uncontrolled proliferation of the intracellular bacterial population is clearly a risk to the host. The dual effect of DNF4/NCR211 may reflect a mechanism to ensure that the rhizobia stay in a properly differentiated state.

Host control of terminal bacteroid differentiation has evolved in multiple lineages of legumes, indicating a possible fitness benefit to the host plant (20). Furthermore, nodules with differentiated bacteroids returned more benefit to the host (20). In an accompanying report, Horváth et al. (28) identified *M. truncatula DNF7* encoding NCR169, suggesting that more than one NCR peptide can be indispensable for the nitrogen-fixing symbiosis. In another companion study, Price et al. (29) recovered a rhizobial peptidase capable of degrading host NCR peptides. This collection of discoveries demonstrates the evolving nature in controlling bacterial differentiation in classical host–microbe mutualism.

Materials and Methods

Plant Materials and Growth Conditions. *M. truncatula* Jemalong ecotype and *dnf4* mutant plants were grown in vermiculite in a growth chamber (22/18 °C, 16-h/8-h day/night) under fluorescent lamps (~100 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Plants were inoculated with *Sinorhizobium medicae* strain ABS7 carrying *pHemA::lacZ* (21), *S. meliloti* Rm1021 carrying *pNifH::uidA* (12), or Rm1021 carrying *pTrp::GFP* (22).

Plasmid Constructs. For promoter-GUS fusion constructs, genomic DNA was amplified with the primers DNF41-F1 (5′-ggatccCGGAGTGTAGGGGTGATGTT-3′) and DNF41-R3 (5′-CAAACTTGAGAATTTCAGCCA-3′) for *NCR211*, as well as DNF42-F10 (5′-ggatccCCTC-TTAAATTGATTAAAGGC-3′) and DNF42-R5 (5′-TTTTTTTTTT-AACTTTGCCTCT-3′) for *NCR178*. The products were cloned into the *pCR8/GW/TOPO* vector (Life Technologies), checked for sequence errors, and transferred to the *pMDC163* binary vector (23) by LR recombination. For the translational fusions of GFP to the C-termini of NCRs, genomic DNA was amplified with primers DNF41-F1 and DNF41-R4 (5′-CTTGGGATAGCTCACA-CAATT-3′) for *NCR211* and DNF42-F10 and DNF42-R6 (5′-GTGGGTACGACAAT-CACAA-3′) for *NCR178*, and then cloned into the *pCR8/GW/TOPO* vector. The Gateway-compatible GFP vector *pK7FWG2-R* (24) was cut with HindIII/SpeI, blunted with Klenow enzyme, and self-ligated to remove the *35S* promoter in front of the Gateway cassette, resulting in *pMK77*. The inserts in the *pCR8/GW/TOPO* vector were checked for sequence errors and then transferred to the *pMK77* binary vector by LR recombination.

Microscopy. For toluidine blue staining, nodules were fixed in 2.5% (vol/vol) glutaraldehyde in 0.05 M cacodylate buffer, washed, dehydrated, and embedded in Technovit 7100 (Heraeus Kulzer), according to the manufacturer's instructions. Then 5-μm sections were cut on a microtome (Reichert-Jung) and stained with 0.05% toluidine blue solution in 1× PBS buffer. Photographs were taken with an Eclipse TE2000-S microscope (Nikon).

Transgenic nodules were selected based on DsRED1 expression using an Olympus SZ61 binocular fitted with an ET590lp optical filter (Chroma

Technology). A Nikon NI-150 illuminator fitted with an ET560/40x optical filter cube (Chroma Technology) was used for excitation of DsRED1.

PI staining was done with hand-sectioned nodules at 30 μM for 20 min. Sections were then briefly washed with water and observed under a confocal microscope (Olympus FluoView FV1000).

The GFP/SYTO9 (Life Technologies) signal was detected by excitation with a 473-nm laser and emission with a 490- to 540-nm bandpass filter. The PI/DsRED1 signal was detected by excitation with a 559-nm laser and emission with a 575- to 675-nm bandpass filter.

SDS/PAGE and Immunoblot Analysis. Nodule samples were harvested from 21-dpi plants. Total protein extracts were prepared by grinding the samples in a 1.5-mL microtube with SDS sample buffer [60 mM Tris·HCl pH 6.8, 60 mM DTT, 2% (wt/vol) SDS, 15% (wt/vol) sucrose, 5 mM ε-amino-*N*-caproic acid, and 1 mM benzamidine]. Protein concentration was measured with a Coomassie blue binding assay (25). Here 10 μg of total nodule proteins were separated by SDS/PAGE and blotted onto a nitrocellulose membrane for immunoblot analysis. The membrane was probed with an antibody against alfalfa leghemoglobin provided by Dr. Carroll Vance (University of Minnesota). A secondary antibody conjugated with horseradish peroxidase was incubated with the membrane. The signal was visualized by enhanced chemiluminescence (Thermo Scientific).

Identification of the *DNF4* Gene. The *dnf4* mutant was isolated from mutagenized *M. truncatula* Gaertn. cv Jemalong seeds created by fast neutron bombardment (12). The *dnf4* mutant was crossed with the A20 ecotype for rough mapping. The location was narrowed down to the upper arm of chromosome 4 between markers 003F07 and h2_133k2b. Then genomic DNA was extracted from a pool of 40 mutant plants isolated from the mapping population and sent to the J. Craig Venter Institute for whole-genome sequencing using an Illumina HiSeq. Visualization of sequence reads was done with the Integrative Genomics Viewer (26). The primers used to amplify across the deletion break point in *esn1* are esn1-LB (5′-GGTCCTGAAGTACCATATCTTAGT-3′) and esn1-RB (5′-GATTAAGTGCAAGTATACAT-TGTCC-3′).

Complementation of the *dnf4* Mutant. Genomic DNA sequences encoding NCR211 (Medtr4g035705) and NCR178 (Medtr4g035725) were amplified and cloned into *pCR8/GW/TOPO* with primers DNF41-F1 and DNF41-R1 (5′-ggatcc-GGAGGGGGTCTAT-GAGAGCA-3′) for NCR211 and DNF42-F1 (5′-ggatcc-GCAGGTTTGACATCCTCACC-3′) and DNF42-R1 (5′- CCTCACTAATCACTGACG-GACC-3′) for NCR178. The cloned inserts were checked for errors and transferred to a binary vector, *pKGW-R* (24). Hairy root transformation was performed with *Agrobacterium rhizogenes* strain ARqua1 harboring the constructs according to the method outlined by Boisson-Dernier et al. (27). More than 10 independent transgenic roots were selected by red fluorescence of the DsRED1 marker gene for scoring of nodule phenotype.

GUS Staining. Roots with nodules were fixed in cold 90% (vol/vol) acetone for 30 min and then rinsed with cold water. Samples were then immersed in 50 mM sodium phosphate buffer (pH 7.2) containing 2 mM 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide cyclohexylammonium salt (X-Gluc), 0.2% Triton X-100, and 2 mM each potassium ferricyanide and ferrocyanide under vacuum for 30 min, followed by further incubation at 37 °C for 2–5 h. The nodules were embedded in 6% (wt/vol) low-melting agarose and sectioned in 70-μm slices with a vibratome (Vibratome 100 Plus). Nodules from more than five independently transformed roots showed the same GUS staining pattern.

Peptide Treatment of Free-Living *S. meliloti*. *S. meliloti* Rm1021 cultures were grown to an OD_{600} of 0.3–0.6 in LB media and washed with 5 mM Mes-KOH buffer at pH 5.8. Then 200 μL of bacteria (diluted to an OD_{600} of 0.1) were treated with peptides (synthesized by GenScript) at the indicated concentrations and incubated for 3–4 h at 30 °C. After the peptide treatment, bacterial suspensions were serially diluted and plated out in triplicate on selective media. Colony-forming units per milliliter were measured after 2 d of incubation at 30 °C.

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