Medicago truncatula symbiotic peptide NCR247 contributes to bacteroid differentiation through multiple mechanisms

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Symbiosis between rhizobia soil bacteria and legume plants results in the formation of root nodules where plant cells are fully packed with nitrogen fixing bacteria. In the host cells, the bacteria adapt to the intracellular environment and gain the ability for nitrogen fixation. Depending on the host plants, the symbiotic fate of bacteria can be either reversible or irreversible. In *Medicago* and related legume species, the bacteria undergo a host-directed multistep differentiation process culminating in the formation of elongated and branched polyploid bacteria with definitive loss of cell division ability. The plant factors are nodule-specific symbiotic peptides. Approximately 600 of them are nodule-specific cysteine-rich (NCR) peptides produced in the rhizobium-infected plant cells. NCRs are targeted to the endosymbionts, and concerted action of different sets of peptides governs different stages of endosymbiont maturation, whereas the symbiotic function of individual NCRs is unknown. This study focused on NCR247, a cationic peptide exhibiting in vitro antimicrobial activities. We show that NCR247 acts in those nodule cells where bacterial cell division is arrested and cell elongation begins. NCR247 penetrates the bacteria and forms complexes with many bacterial proteins. Interaction with FtsZ required for septum formation is one of the host interventions for inhibiting bacterial cell division. Complex formation with the ribosomal proteins affects translation and contributes to altered proteome and physiology of the endosymbiont. Binding to the chaperone GroEL amplifies the NCR247-modulated biological processes. We show that GroEL1 of *Sinorhizobium meliloti* is required for efficient infection, terminal differentiation, and nitrogen fixation.

bacterial targets | antimicrobial peptides | protein interactions | translation inhibition | host peptides

Symbiosis between microbes and hosts, like legumes and various insects, often results in the formation of symbiotic organs where host cells harbor thousands of endosymbiotic bacteria. In rhizobium–legume symbiosis, rhizobia in the soil interact with their host legume and induce the formation of root nodules where bacteria reside in the host cytosol in membrane-enclosed vesicles called symbiosomes. The bacteria adapt to the endosymbiotic lifestyle in the plant cells and gain the capacity for nitrogen fixation, thereby ensuring the nitrogen need of the host plant.

In addition to the physiological changes required for nitrogen fixation, in certain rhizobium–legume interactions, like in the *Medicago truncatula–Sinorhizobium meliloti* symbiosis, the bacteria undergo an additional remarkable differentiation process (1). The endosymbiotic bacteria called bacteroids become polyploid, noncultivable elongated, and frequently branched cells (2). This terminal bacterial differentiation is provoked by hundreds of nodule-specific cysteine-rich (NCR) host peptides that are exclusively expressed in the rhizobium-infected symbiotic cells (3).

Nodule development and bacterial infection are triggered by bacterial signals, the Nod factors as a result of transcriptional activation of *nod* operons induced by NodD and host plant flavonoids (4). In the infected nodules, bacteroid differentiation occurs progressively over different host cell layers and in coordination with the differentiation of these host cells (1). *Medicago* nodules contain a persistent apical meristem (zone I), a 10- to 15-cell layer wide infection zone (zone II), a two- to three-cell layer wide interzone (zone II-III), and a growing nitrogen-fixing zone (zone III). The bacteria infect and multiply in the youngest, nondividing cells below the meristem, whereas in somewhat older cells of zone II, they stop dividing and start differentiating. The most drastic changes occur in the interzone II-III where a sudden growth of bacteroids is visible. In zone III, the bacteroids fill up the cytosol of the host cells in an organized manner. The terminally differentiated nitrogen fixing bacteroids often have a reduced metabolism and when isolated from the nodules, *ex planta* they exhibit increased membrane permeability and are incapable to produce offspring (3, 5).

In *M. truncatula,* close to 600 genes code for NCR peptides (6). These peptides are targeted to the bacteroids via the secretory pathway and are essential for bacteroid differentiation because blocking the delivery of symbiotic peptides to the bacteroids abolished the bacteroid differentiation completely (3). The *NCR* genes are small and usually composed of two exons: the first one codes for a conserved signal peptide and the second one for the mature active peptide. The NCRs have four or six cysteine residues at conserved positions and exhibit high divergence in their amino acid compositions and sequences resulting in a wide range of cationic, neutral, and anionic peptides (7).

Significance

Intracellular endosymbiotic bacteria in diverse symbiotic systems are under the control of host-derived symbiosis-specific peptides. These peptides have mostly unknown activities. In the facultative rhizobium-legume symbiosis, the bacteria differentiate in many legumes to large polyploid noncultivable bacteroids. This terminal differentiation is achieved by concerted actions of hundreds of nodule-specific cysteine-rich (NCR) peptides. Although in vitro antimicrobial activities were demonstrated for a few NCR peptides, their mode of action *in planta* remained unexplored. Our work reveals a complex interaction network of NCR247, which affects bacterial cell division machinery, translation, and protein folding. These findings give an insight how the host can modulate the physiology of the endosymbionts and may serve as a paradigm for other symbiotic systems.

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NCRs resemble defensins, the major class of antimicrobial peptides of plant innate immunity, which have eight cysteine residues and a different signal peptide.

Different sets of the *NCR* genes are activated during the early and later stages of bacteroid differentiation, and combined action of the NCR peptides is needed for the progressive differentiation events. The hitherto-tested NCRs showed colocalization with the bacteroids and were present in the bacterial membranes, or in the cytosol (3).

The high divergence of NCRs in amino acid composition, charge, and sequence means likely that the peptides have different modes of action and bacterial targets. We have shown that chemically synthesized cationic NCR peptides have antibacterial activity in vitro, causing increased permeability of the bacterial membranes and ultimately cell death (3, 8, 9). This study is focused on one of these cationic peptides, NCR247, a 24-aa-long peptide based on its remarkable hypothetic binding properties. We addressed the following questions: (*i*) which stage of symbiotic cell development is associated with expression of NCR247, (*ii*) how NCR247 interacts with the bacterial cells, (*iii*) whether the NCR247 peptide is present in the bacteroids and, if so, (*iv*) what are its bacterial targets and which bacterial functions are affected.

Results

Sequence Analysis of NCR247 Predicts Exceptional Protein Binding Properties. Analyzing in silico the hundreds of NCR peptides, NCR247 appeared to have distinct properties from the others. The amino acid sequence of NCR247 is unique, not closely homologous to any known protein. Distant similarities between NCR247 and various proteins were found (*SI Appendix*, Fig. S1) including proteins that destruct cells (defensins, toxins) or bind to other proteins. The composition of NCR247 strongly deviates from an even distribution of amino acids, providing an overall cationic polar feature. The hydropathy index (also known as the Boman index) is a numeric value relevant to biological activity and estimates the binding ability of antimicrobial peptides to other proteins (10). This parameter was determined for NCR247, and for all proteins present in UniProtKB/Swiss-Prot database. The hydropathy value of NCR247 is 4.63 kcal/mol, the highest among NCR peptides, and also higher than 99.86% of all known proteins including the two multifunctional antimicrobial peptides PR-39 and LL-37 (index values of 3.04 and 3.00, respectively) (*SI Appendix*, Table S1*A*). The calculated extreme hydrophilic character indicated the ability of NCR247 to exert multiple activities.

NCR247 Expression Coincides with Differentiation of Endosymbionts. Transcriptome analysis of nodules at different stages of development indicated induction of NCR247 in the early stages of nodule development (11). To localize precisely the gene expression in the nodule, an *NCR247* promoter-driven translational fusion with the β-glucuronidase (GUS) reporter gene was introduced into *M. truncatula* with hairy root transformation. GUS activity corresponding to *NCR247* expression in transgenic nodules was present in the older cell layers of nodule zone II, where the endosymbionts stop dividing and cell elongation begins, and in the interzone II-III, where dramatic growth of endosymbionts occurs (Fig. 1*A*).

NCR247 Penetrates the Bacterial Membranes and Accumulates in the Cytosol of Bacteroids. Cationic antimicrobial peptides (AMPs) can interact with microbial membranes and form pores that lead ultimately to lysis and death of the cells (12). In vitro NCR247 has also antimicrobial properties (with a minimal inhibitory concentration of 5 μM on *S. meliloti*) allowing entry of the membrane impermeable dye propidium iodide (PI) into the bacteria and causing cell death (3, 9). However, in the nodules, the bacteroids' membranes are impermeable for PI, suggesting that the peptide concentrations might be significantly lower in the nodule cells (8). For imaging the localization of NCR247 in the free-living bacteria and in the bacteroids, the N terminus of NCR247 was labeled with fluorescein isothiocyanate (FITC), which did not alter the peptide's hydrophobicity and activity (*SI Appendix*, Table S1*B* and Fig. S2). FITC-NCR247 was added to log phase *S. meliloti* cultures (OD_{600} = 0.5) at sublethal 1.5 μM concentration. In addition, the bacterial cells were stained either with PI for detection of membrane damage or with FM 4-64, which stains the bacterial membranes. Fluorescent labeling of the cells was observed over a 1-h period with confocal microscopy. FITC itself did not result in any labeling or background signal in the bacteria. The cells treated with FITC-NCR247 and stained with PI showed only the FITC fluorescent signal in the bacteria (Fig. 1*B*). The molecular mass of PI (668.4 g/mol) is fivefold less than that of FITC-NCR247 (3,399 g/mol), thus the absence of PI label indicates that NCR247 at sublethal concentrations does not make such membrane alterations that are required for PI entry, while itself can either go through the bacterial membranes or it is taken up by the bacterial cell. Labeling with the membrane-specific FM 4-64 fluorescent dye partially overlapped with FITC-NCR247, although the major site of the peptide accumulation was in the bacterial cytoplasm (Fig. 1*C*). When FITC-NCR247 and FM 4-64 were added to freshly isolated differentiated bacteroids, there was no overlap between the FITC and FM 4-64 labeling; FM 4-64 marked the membranes, whereas FITC-NCR247 was only present in the cytosol in uneven distribution, accumulating in numerous nucleoid-like globular subdomains (Fig. 1 *D* and *E*).

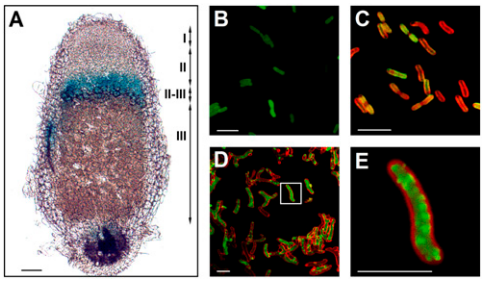

Fig. 1. NCR247-GUS expression in nodules and localization of FITC-NCR247 in *S. meliloti* bacteria and bacteroids. (*A*) Blue staining in the older cells of zone II and in the interzone II-III corresponds to NCR247-GUS expression in transgenic *M. truncatula* nodules. I, nodule meristem; II, infection zone; II-III, interzone; III, nitrogen fixation zone. (*B*) *S. meliloti* culture stained with FITC-NCR247 and PI. (*C*) *S. meliloti* culture stained with FITC-NCR247 and FM 4-64. (*D* and *E*) *S. meliloti* bacteroids stained with FITC-NCR247 and FM 4-64. (*E*) Enlarged image of the boxed bacteroid from *D*. (Scale bars: 5 μm.)

NCR247 Has Multiple Intracellular Bacterial Targets in Free-Living Bacteria as Well as in Bacteroids. The localization of NCR247 in the endosymbionts suggested that NCR247 might have specific bacterial targets and may modulate the bacterial physiology. To identify the intracellular targets of NCR247, the peptide was marked during the synthesis with the StrepII (WSHPQFEK) or with FLAG (DYKDDDDK) tag at the C terminus that made possible isolation of NCR247 together with its interacting partners on Strep-Tactin or FLAG-affinity (M2 agarose) columns. The StrepII and FLAG tags alone had no detectable antimicrobial activity under our experimental conditions (*SI Appendix*, Fig. S2) and, coupled to NCR247, they had minor effects either on the charge (NCR247, pI: 10.15 vs. NCR247-StrepII, pI: 10.06, or NCR247-FLAG, pI: 9.11), or on the hydropathy profile (*SI Appendix*, Table S1*A*) and on the in vitro antimicrobial activity (*SI Appendix*, Fig. S2). NCR247-StrepII or NCR247-FLAG added at 3 μM sublethal concentration to *S. meliloti* bacterial cultures or directly to a crude extract of *S. meliloti* for 2 h resulted in comparable pattern and complexity of interacting bacterial proteins, whereas the StrepII or the FLAG tag alone did not bind bacterial proteins (representative samples are shown in Fig. 2 *A* and *B*). Because the physiology of cultured bacteria and of endosymbiotic bacteroids is not similar, we investigated whether NCR247-StrepII interacts with the same partners in the bacteroids as in the free living bacteria. Affinity chromatography of NCR247-StrepII–treated bacteroid extracts resulted in an altered pattern

with reduced complexity and abundance of the NCR247-StrepII–interacting proteins (Fig. 2*C*). This difference was consistent with the significantly different composition of the protein extracts of *S. meliloti* cultures and bacteroids because the latter exhibited a lower diversity of proteins and, remarkably, diminished quantity of proteins from ∼30–10 kDa (Fig. 2*D*).

Expression of NCR247 in the older cells of nodule zone II raised the possibility that NCR247 might be involved in preventing multiplication of the endosymbiont. FtsZ is a conserved bacterial cell cycle protein required for septum formation and cell division (13). Because the polyploidy of bacteroids must result from the inhibition of cell division, we tested whether NCR247 is capable to interact with FtsZ. Western blot analysis of both the NCR247-StrepII and NCR247-FLAG eluates with anti-FtsZ antibody demonstrated the presence of FtsZ among the interacting proteins (Fig. 2 *E* and *F*). Cytoplasmic localization of NCR247 and its interaction with FtsZ suggested that NCR247 may bind to the FtsZ monomers and inhibits FtsZ polymerization, thus Z-ring and septum formation. To test this hypothesis, log-phase *S. meliloti* cultures were grown in the presence of 2.5 μM NCR247, allowing two rounds of replication in the control cultures, and then the presence of septum was visualized by the addition of FITC-NCR035 that marks the septum and bacterial membranes (3). In the control cultures as expected, FITC-NCR035 was present in the membrane and accumulated in the septum (*SI Appendix*, Fig. S3*A*). In contrast, the septum labeling was missing in the NCR247-treated cultures (*SI Appendix*, Fig. S3 *B* and *C*), whereas significant elongation of bacterial cells could be observed (*SI Appendix*, Fig. S3*C*), supporting a role for NCR247 in cell division arrest via its interaction with FtsZ. In the eluate of NCR247-StrepII–treated bacteroids, FtsZ was undetectable (Fig. 2*G*), which raised the possibility that bacteroids with definitive loss of their cell division ability do not produce FtsZ. We show that FtsZ is absent in the bacteroids (Fig. 2*H*).

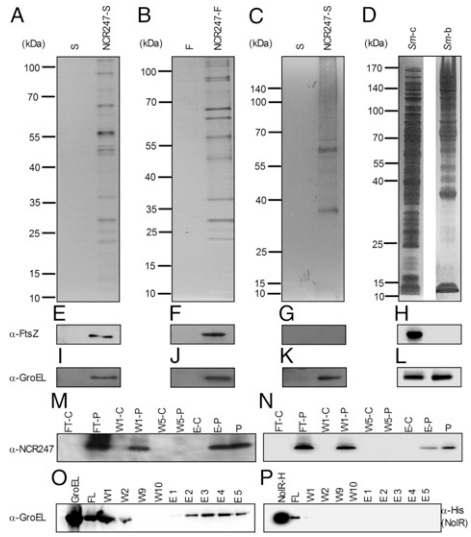

Fig. 2. NCR247-interacting proteins in S. *meliloti* bacteria and bacteroids. (*A*) SDS/PAGE and silver staining of the elution fractions from Strep-Tactin column of StrepII (S) and NCR247-StrepII (NCR247-S) treated *S. meliloti* bacterium extracts. (*B*) SDS/PAGE and silver staining of samples eluted from anti-FLAG beads from FLAG (F) and NCR247-FLAG (NCR247-F) treated *S. meliloti* bacterium extracts. (*C*) SDS/PAGE and silver staining of samples eluted from Strep-Tactin column of StrepII tag (S) and NCR247-StrepII (NCR247-S) treated *S. meliloti* bacteroid extracts. (*D*) SDS/PAGE and silver staining of extracts from *S. meliloti* bacteria (Sm-c) and bacteroids (Sm-b). (*E–H*) Detection of FtsZ in the NCR247 complexes of *A–C* and in the *S. meliloti* bacteria and bacteroid extracts (*D*) with anti-FtsZ antibodies. (*I–L*) The presence of GroEL in the NCR247 complexes shown in *A–C* and in *S. meliloti* bacteria and bacteroids shown in *D*. (*M* and *N*) Anti-GroEL affinity chromatography confirms the presence of NCR247 in the GroEL complex of *S. meliloti* bacteria (*M*) and bacteroids (*N*) with anti-NCR247 antibodies. (*O*) Binding of NCR247-StrepII to purified GroEL. GroEL detected with anti-GroEL antibodies is present in the elution fractions. (*P*) NCR247-StrepII does not bind to purified NolR-6xHis (NolR-H) because NolR-H is undetectable in the elution fractions. C, control; E, elution and the numbers indicate the elution fractions; FT, flow through; P, NCR247; W, washing and the numbers indicate the washing steps. Purified GroEL and NolR-H were used at 0.5 μM as controls for detection.

For identification of the multiple NCR247-interacting proteins, the protein bands were either cut out of the gels or the eluted fractions from the affinity columns without further separation were subjected to LC-MS/MS analysis. The list of proteins identified in the NCR247 complexes is shown in *SI Appendix*, Table S2*A*. In the extracts of cultured bacteria treated with either NCR247-StrepII or NCR247-FLAG, the ribosomal proteins formed the majority; altogether 14 small subunit (S1, S2, S3, S4, S5, S6, S7, S8, S9, S11, S12, S13, S16, S18) and 12 large subunit (L1, L2, L3, L4, L5, L6, L9, L10, L13, L16, L19, L23) proteins were identified. In addition, the GroEL bacterial chaperone proteins were highly abundant and commonly present as well as the pyruvate dehydrogenase complex, transaldolase, DNA-directed RNA polymerase subunits β and β′, elongation factor Tu and G, and a Maf-like protein. In the bacteroids, GroEL, the pyruvate dehydrogenase complex, and, in lesser extent, ribosomal proteins were also part of the NCR247 complexes (*SI Appendix*, Table S2*B*). The presence of GroEL in the NCR247 complexes was confirmed with Western blot analysis of the eluates of bacterial and bacteroid extracts as well as in the bacteria and bacteroids (Fig. 2 *I–L*). In the bacteroids, additional bacteroid-specific binding partners of NCR247 were detected, namely subunits of the nitrogenase complex but also three other NCR peptides (NCR028, NCR169, and NCR290), indicating that these host peptides are present in the bacteroids and might form heterocomplexes.

NCR247 Inhibits Bacterial Protein Synthesis. Although the ribosomal proteins are known targets of antibiotics, their high abundance and sticky nature led us to question the biological significance of their interactions with the NCR247 peptide. However, the altered pattern and reduced complexity of bacteroid proteins (Fig. 2*D*) suggested that the proteome of the bacteroids might be influenced by the NCR peptides. The effect of NCR247 on translation was tested in two prokaryotic cell-free transcription-translation systems: one driving the production of the green fluorescent protein (GFP) from the T7 promoter and the second one the luciferase production from the bacterial hybrid *tac* promoter using the T7 bacteriophage RNA polymerase and the bacterial RNA polymerase, respectively. In addition to NCR247, NCR247-StrepII, the StrepII tag alone, and NCR001, produced in the mature symbiotic cells (7), were also tested in various concentrations ranging from 1 to 500 μM. As controls H_2O, streptomycin, a known prokaryotic translation inhibitor acting on the S12 ribosomal protein, and the transcription inhibitors actinomycin D or rifampicin were used. Translation of the GFP protein was followed by measuring fluorescence intensity (Fig. 3*A*). H_2O, NCR001, or the StrepII tag had no or only moderate effect on progressive accumulation of GFP, whereas NCR247 and NCR247-StrepII strongly reduced, and streptomycin and actinomycin D entirely blocked, GFP translation. The minimal inhibitory concentration of NCR247 in the concentrated lysate was 100 μM. Similarly, in the second cell-free system, translation of luciferase measured by luminescence intensity was strongly inhibited by NCR247 and NCR247-StrepII and completely abolished by streptomycin, rifampicin, and actinomycin D (Fig. 3*B*). We also tested how NCR247 affects protein synthesis in growing *S. meliloti* cultures by monitoring the incorporation of ^{3}H-leucine into proteins. Using different concentrations (0.5, 1, and 5 μM) of NCR247, a dose-dependent decrease of protein synthesis was observed in vivo even at sublethal concentrations (Fig. 3*C*). Together, these in vitro and in vivo results suggest that one mode of the NCR247 peptide actions is binding to the ribosomes and, thereby, negatively affecting translation.

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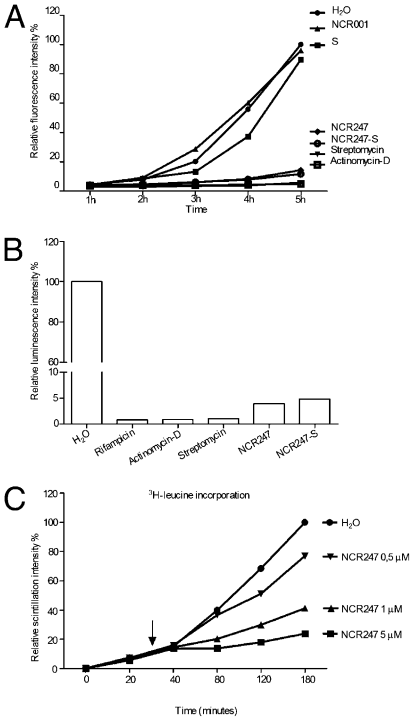

Fig. 3. NCR247 inhibits bacterial translation. (*A*) In vitro translation of GFP measured with GFP fluorescence in the presence of H_2O, NCR247, NCR247-StrepII (NCR247-S), StrepII tag (S), NCR001, streptomycin, and actinomycin-D, all applied at 100 µM. (*B*) In vitro translation of luciferase measured via luminescence intensity after 30-min incubation in the presence of NCR247, NCR247-S, streptomycin, actinomycin-D, or rifampicin, all at 100 µM. (*C*) Incorporation of ^{3}H-leucine into *S. meliloti* proteins at different concentrations of NCR247. NCR247 was added 30 min after reaction initiation (arrow).

GroEL Is Essential for Differentiation of Nitrogen Fixing Bacteroids. GroEL was a major interactor of NCR247 both in cultured bacteria and bacteroids (Fig. 2 *I–L* and *SI Appendix*, Table S2 *A* and *B*). However, because GroEL is a highly abundant bacterial protein that interacts with hundreds of proteins (14), it raised the possibility that GroEL–NCR247 interaction may have no biological relevance. To exclude that NCR247 sticks nonspecifically to abundant proteins, we checked whether the most highly expressed bacterial proteins (based on transcriptome data) were among the NCR247-interacting partners (listed in *SI Appendix*, Table S3). None of the protein products of these abundant transcripts, including two ribosomal proteins, were identified in the NCR247 complexes. Then we tested whether immunoprecipitation (IP) of GroEL with anti-GroEL antibody will recover NCR247 from the NCR247-StrepII–treated extracts of bacteria (Fig. 2*M*) and bacteroids (Fig. 2*N*). NCR247 detected with anti-NCR247 antibodies was present in the elution of NCR247-StrepII–treated bacterial and bacteroid extracts. To further support specific interaction of NCR247 with GroEL, we performed IP experiments using NCR247-StrepII with purified GroEL and NolR, a transcriptional regulator from *S. meliloti* expressed at a fourfold higher level than GroEL (15). Both proteins and NCR247-StrepII were used at 10 µM concentrations. GroEL formed a complex with NCR247-StrepII and was present in elution fractions (Fig. 2*O*), in contrast to NolR marked with 6xHis tag and detected with anti-6xHis antibody that was absent in the eluates (Fig. 2*P*). The IP experiments unambiguously demonstrated that NCR247 binds GroEL but does not interact with NolR. These results collectively confirmed the existence of GroEL–NCR247 complex(es) and raised the possibility that GroEL might be necessary for correct folding of the NCR peptides, or NCR247 by its interaction with GroEL affects GroEL-dependent functions.

Therefore, we investigated how GroEL deficiency influences the fate of bacteroids and nodule differentiation. *S. meliloti* has five *groEL* genes, and previous studies showed that the presence of *groEL1* is essential for symbiosis because the *groEL1* single mutant could not fully induce *nod* gene expression and formed ineffective nodules on *Medicago sativa* (16). Because the effect of GroEL1 deficiency has not been explored further, we studied the development of symbiotic cells and particularly the fate of bacteroids in nodules formed on *M. sativa* and *M. truncatula* by quadruple *groEL* mutants of *S. meliloti*-containing functional GroEL1 (strain AB238) or GroEL2 (strain AB257). Both on *M. sativa* and *M. truncatula*, AB238 produced elongated pink nitrogen fixing nodules like the wild-type strain 1021, whereas AB257 formed small white spherical nodules (shown for *M. sativa* in Fig. 4 *A*, *E*, and *I*). Nodule sections stained with Syto9 were investigated with confocal microscopy. Nodule structure, formation of symbiotic cells, and differentiation of bacteroids were similar in nodules induced by the wild-type strain 1021 and strain AB238 (Fig. 4 *B–D* and *F–H*). The white nodules induced by AB257 showed a diversity of phenotypes in bacterial infection and differentiation of the bacteroids. Certain nodules were completely devoid of bacteria (Fig. 4 *J* and *K*), but the majority of nodules contained a few infected cells (Fig. 4 *M* and *N*). In these symbiotic cells, the AB257 bacteria either remained undifferentiated (Fig. 4*L*) or differentiated to some extent to elongated cell type without attaining full differentiation (Fig. 4*O*). Both undifferentiated and elongated AB257 bacteroids were present in lower density in the infected cells than the wild-type or the AB238 strain. Moreover, despite the elongation of certain AB257 bacteroids, no nitrogen fixation was observed, leading to early death of plants (*SI Appendix*, Fig. S4). These results indicate a constant need for GroEL at different stages of nodule development, including cellular infection, differentiation, maintenance, and nitrogen fixation of endosymbionts.

Discussion

NCRs represent a recently discovered class of peptides with unresolved biological activities that only exist in *Medicago* and a few closely related legume species. NCRs are essential for differentiation and maintenance of their bacterial endosymbionts and likely evolved from innate immunity antimicrobial peptides. In their natural settings, NCRs do not kill the bacteria during the symbiotic interaction. Here we show that the cationic NCR247

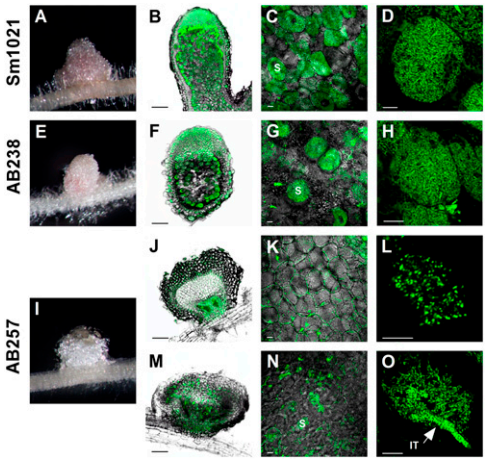

Fig. 4. Requirement for GroEL1 in nitrogen-fixing nodule development. *M. sativa* nodules induced by Sm1021 (*A–D*), AB238 harboring only GroEL1 (*E–H*), and AB257 having only GroEL2 (*I–O*). Nitrogen-fixing elongated pink nodules (*A* and *E*) and small white spherical nonfixing nodule (*I*). (*B*, *F*, *J*, and *M*) nodule sections stained with Syto9. (*C*, *G*, *K*, and *N*) images from the central nodule regions. S, symbiotic cells. (*D*, *H*, *L*, and *O*) Enlarged image of symbiotic cells. IT, infection thread containing multiplying undifferentiated bacteria (arrow).

peptide acts in those nodule cells where bacterial cell division is arrested and elongation of bacteroids occurs. Our work sheds light on the multifaceted interaction of NCR247 with its symbiotic *S. meliloti* partner from combined in vitro and *in planta* investigations of this peptide.

Involvement of NCR247 in Cell Division Arrest and Filamentous Growth. The expression pattern of NCR247 in the older zone II cells and in the interzone II-III suggested that NCR247 might interfere with the bacterial cell division and provoke cell elongation. In rod-shaped bacteria, after DNA replication and cell elongation, a Z ring is formed by localization and polymerization of FtsZ around the longitudinal midpoint of the cell. The Z-ring establishes the position of the future septum and serves as a scaffold for the formation of the divisome that completes septation (13).

Here we show that FtsZ forms a complex with NCR247. NCR247 is present in the cytosol of bacteria, but it has not been localized specifically to the septum, unlike NCR035, which is associated with the initiation and formation of the septum (3). Expression of NCR247 and NCR035 overlaps in the nodule zone II and both of them—together with other NCRs—act in the same symbiotic cell. Based on the homogenous distribution of NCR247 inside bacterial cells NCR247 interacts with FtsZ before its localization to the cell midpoint, whereas NCR035 may act subsequently, affecting the septum assembly. NCR247 likely binds to the FtsZ monomers and inhibits their polymerization because treatment of *S. meliloti* cells with NCR247 abolished septum localization of NCR035 and the absence of septum provoked significant cell elongation. In line with this observation, Penterman et al. showed recently that FtsZ-GFP–labeled Z-ring formation was strongly reduced by NCR247 treatment in *S. meliloti* (17). Because nodule cells can accommodate only limited numbers of bacteria, inhibition of endosymbionts' multiplication in the older zone II cells might be crucial for the host and likely achieved by individual and concerted action of several NCRs, acting on different steps of the same pathway in the same symbiotic cells.

We have shown that the FtsZ protein is absent in the bacteroids. This finding is consistent with the incapacity of bacteroids for cell division. *S. meliloti* strains contain minimum three replicons (the chromosome and two megaplasmids and, depending on the strains, additional smaller plasmids) that are amplified at least 20-fold in the bacteroids and distributed in multiple nucleoids of various sizes in a seemingly unorganized manner (2). Such organization of multiple replicons makes rather unlikely that septum formation could lead to equal repartitioning of the haploid genome to daughter cells and could be the cause of irreversible loss of cell division ability. In the bacteroids, NCR247 localized to globular structures of various sizes that resemble the multiple nucleoids but could also be nucleoid-associated ribosomal complexes.

NCR247–Ribosomal Protein Interactions: Modulation of the Bacterial Protein Synthesis. The pulldown experiments with the tagged NCR247 peptides identified a high number of ribosomal proteins that interacted with NCR247. These interactions could be direct but, more likely, binding of one or a few primary ribosomal target proteins attracts additional ribosomal proteins or even the whole ribosome. Addition of NCR247 to *S. meliloti* cultures or to in vitro translation systems slowed down or fully inhibited protein synthesis in a concentration-dependent manner. In the bacteroids, the protein synthesis is ongoing, but the complexity of the proteome is reduced; a large portion of proteins of <30 kDa is diminished, whereas others like the nitrogenase and GroEL are produced in high quantities. Interestingly, NCR247 attracted fewer ribosomal proteins from the bacteroid extracts. The molecular mass of ribosomal proteins ranges from ∼8 to 32 kDa except for S1, which is ∼63 kDa in *S. meliloti* (18). The reduced number of proteins below 32 kDa and fewer ribosomal interacting partners of NCR247 in the bacteroids were indicative for reduced amounts of ribosomal proteins and even diversification of the ribosomes, comprised of lower amounts of certain ribosomal proteins. Evidence is accumulating that ribosomal heterogeneity represents another level of complexity in bacterial translation regulation (19). Comparing the transcriptome of *S. meliloti* bacteria and bacteroids reveals at least 10-fold lower expression level of ribosomal protein genes in the bacteroids (*SI Appendix*, Fig. S5*A*) and differences in the relative transcript abundance of the various ribosomal proteins (*SI Appendix*, Fig. S5*B*). RNA-Seq analysis of *S. meliloti* cultures treated with sublethal doses of NCR247 resulted in down-regulation of genes coding for ribosomal subunits (9), which might indicate contribution of NCR247 to ribosome diversification in the bacteroids.

Versatile Requirement for GroEL in Symbiosis. Of the five *groEL* genes in *S. meliloti*, either *groEL1* or *groEL2* alone is sufficient for survival, whereas only *groEL1* is essential for symbiosis. In contrast to the wild-type elongated pink nitrogen fixing nodules, the *groEL1* mutant induced small, spherical, and white nodules that did not fix nitrogen (15). Lack of nitrogen fixation was the consequence either of the total absence of endosymbionts or the insufficient number of infected cells containing low density of bacteroids. Bacterial infection requires Nod factor production (4). GroEL was shown to be required for efficient binding of NodD to the *nod* promoters and full activation of the *nod* regulon (20). In the absence of GroEL1, Nod factor production appears to be sufficient for nodule induction but not for the infection process. Expression of the NCR genes requires cellular infection and the presence of some bacteria in the symbiotic nodule cells (21). Thus, in the infected nodules, the early NCRs are probably produced; however, the existence of nodules containing only undifferentiated bacteroids indicates that GroEL1 might be needed for NCR activities and terminal differentiation of bacteroids. In a portion of the nodules induced by the *groEL1* mutant, the endosymbionts differentiated to elongated bacteroids; however, the nodules were not functional and nitrogen fixation activity was not detected. GroEL is required for nitrogenase assembly in other bacteria (22). Thus, likewise, in the absence of GroEL1, subunits of nitrogenase may not form an active enzyme complex. The NCR247 complex isolated from the bacteroids contained both the nitrogenase subunits and GroEL. Although a direct interaction between the nitrogenase and NCR247 cannot be excluded, the nitrogenase can also be part of the NCR247 complex via GroEL. Similarly, many other proteins in the NCR247 complexes are potential GroEL substrates such as the ribosomal proteins, DNA-directed RNA polymerase, elongation factors, or pyruvate dehydrogenase subunits (11). Although our data does not provide unequivocal evidence for direct binding of these proteins, interaction of NCR247 with GroEL represents a multifunctional way to manipulate essential bacterial functions contributing to the bacteroid differentiation process.

Host Peptides and GroEL: General Actors in Microbe–Host Interactions. The involvement of host antimicrobial peptides in controlling the endosymbionts' multiplication and fate is not unique for the rhizobium–legume symbiosis. In insects of the *Sitophilus* genus (weevils), the symbiotic cells called bacteriocytes contain giant filamentous endosymbionts and produce antimicrobial peptide coleoptericin-A (ColA) (23). Silencing the *colA* gene resulted in reduced elongation of the endosymbionts and their spreading from the bacteriocytes into insect tissues, indicating that ColA is essential for symbiont differentiation regulating their growth through inhibition of cell division (23). In the bacteriocytes of aphids, expression of a unique class of genes encoding small proteins with signal peptides was discovered that was often cysteine-rich, being reminiscent of NCRs, and named bacteriocyte specific cysteine-rich peptides (24). Similarly, in another insect, the stinkbug *Riptortus pedestris*, cysteine-rich peptides are specifically produced by a gut-derived symbiotic organ carrying extracellular bacterial symbionts (25). Although the functions of these aphid and stinkbug peptides are entirely unknown, it is interesting that legume and insect symbiotic cells evolved similar, but not homologous types of peptides, that are specifically produced in symbiotic organs.

Moreover, GroEL is also an essential symbiosis factor in the insects required for endosymbionts maintenance or counteracting the negative effects of deleterious mutations arising from genome erosion (26). Far Western blotting experiments revealed interaction of ColA with GroEL and proposed to be involved in cell elongation of the endosymbiont (23). Although the precise action of host peptides and GroEL needs to be discovered, long-term host–symbiont coevolution might have evolved common strategies in the plant and animal kingdoms.

Materials and Methods

Synthesis of NCR247 Peptides. NCR247 and C-terminal tagged forms (NCR247-StrepII fusion with Strep-tag II; NCR247-FLAG fusion with FLAG tag) and N-terminal FITC labeled NCR247 (FITC-NCR247) were synthesized by conventional solid phase peptide synthesis at >95% purity.

Bacterial Strains, Growth Conditions, and Treatments. *S. meliloti* 1021 and the quadruple *groEL* mutants of strain 1021 carrying either *groEL1* (AB238) or *groEL2* (AB257) (15) were used. For NCR247 treatments, log-phase cultures in 20 mM potassium-phosphate buffer (KPB) at pH 7.0 and OD_{600} = 0.1 were incubated with peptides from 0.1 to 500 μM for 2 h.

Plant Growth, Inoculation, and Transformation. *M. truncatula* (A17) and *M. sativa* (cv. Anna) were grown and inoculated for nodulation with *S. meliloti* strains according to the *Medicago truncatula* Handbook (www.noble.org/MedicagoHandbook). Bacteroids were isolated as described (27). The translational NCR247-GUS fusion containing a 1-kb promoter region until the first codon of the second exon of the *NCR247* gene in pCAMBIA1305.2 vector in place of the 35S promoter was introduced into *M. truncatula* with hairy root transformation using *Agrobacterium rhizogenes* strain A4 (28).

Affinity Purification of Protein Complexes. Intact cells or crude extracts of log-phase *S. meliloti* bacteria concentrated to OD_{600} = 2.0 in KPB or freshly isolated bacteroids in KPB were treated with NCR247-StrepII or NCR247-FLAG at 3 μM final concentration for 2 h. Cleared cell extracts (0.5 mL) were incubated with 200 μL of Strep-Tactin or M2 agarose used for FLAG purification for 1 h at 20 °C. Columns were washed eight times, and the bound proteins were eluted with 2.5 mM desthiobiotin or with 200 μM FLAG peptide. The flow-through, washing, and elution fractions were separated in 10% (wt/vol) or 4–12% (wt/vol) gradient SDS polyacrylamide gels. Protein bands were visualized with silver staining.

Immunoprecipitation with GroEL and NolR. NCR247-StrepII was incubated for 2 h with purified *Escherichia coli* GroEL and *S. meliloti* NolR-6xHis, respectively (all were applied at 10 μM). Affinity purification was performed on Strep-Tactin column. GroEL was detected with anti-GroEL antibody, whereas NolR-6xHis was detected with anti-polyhistidine antibody.

Protein Identification by Mass Spectrometry. Details can be found in *SI Appendix, SI Materials and Methods*.

In Vitro Translation Reactions. In vitro translation reactions were performed with the Rapid Translation System RTS 100 *E. coli* HY Kit (Roche) and with the *E. coli* S30 Extract System for Circular DNA (Promega) in the presence of 1, 10, 50, 100, 250, and 500 μM NCR247/NCR247-StrepII/NCR247-FLAG and various controls (StrepII tag, NCR001, streptomycin, rifampicin, actinomycin D). GFP fluorescence was measured in a Fluostar optima plate reader (BMG Labtech). Luminescence (luciferase activity) was measured in a Lumistar optima plate reader (BMG Labtech).

^{3}H-Leucine Incorporation in *S. meliloti* Bacteria. Fifteen microliters of ^{3}H-Leucine (58.5 Ci/mmol, 1 μCi/μl; PerkinElmer) was added to 650 μL of log-phase *S. meliloti* cultures grown and diluted in LSM medium (29) to OD_{600} = 0.1. Thirty minutes later, NCR247 at 0.5, 1, and 5 μM concentrations and streptomycin at 5 μM were added to the cultures. Samples (100 μL each) taken at 40-min intervals were precipitated with 5 mL of ice-cold 10% trichloroacetic acid on ice for 1 h and collected on 0.22-μm GSTF filters (Millipore). The radioactivity was quantified with scintillation counting.

Microscopy. Histochemical GUS staining in transgenic *M. truncatula* nodules was performed as described (30). An Olympus Fluoview FV1000 confocal laser scanning microscope was used for localization of FITC-NCR247 in *S. meliloti* bacteria and bacteroids and for investigating symbiotic cell development and bacteroid differentiation in nodule sections. Specimens were costained with 750 nM PI, 800 nM FM4-64, or 2 μM Syto9.

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1. Kondorosi E, Mergaert P, Kereszt A (2013) A paradigm for endosymbiotic life: Cell differentiation of Rhizobium bacteria provoked by host plant factors. *Annu Rev Microbiol* 67:611–628.
2. Mergaert P, et al. (2006) Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium-legume symbiosis. *Proc Natl Acad Sci USA* 103(13):5230–5235.
3. Van de Velde W, et al. (2010) Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science* 327(5969):1122–1126.
4. Schultze M, Kondorosi E, Ratet P, Buiré M, Kondorosi A (1994) Cell and molecular biology of rhizobium-plant interactions. *Int Rev Cytol* 156:1–75.
5. Terpolilli JJ, Hood GA, Poole PS (2012) What determines the efficiency of N(2)-fixing Rhizobium-legume symbioses? *Adv Microb Physiol* 60:325–389.
6. Zhou P, et al. (2013) Detecting small plant peptides using SPADA (Small Peptide Alignment Discovery Application). *BMC Bioinformatics* 14:335.
7. Mergaert P, et al. (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(1):161–173.
8. Haag AF, et al. (2011) Protection of Sinorhizobium against host cysteine-rich antimicrobial peptides is critical for symbiosis. *PLoS Biol* 9(10):e1001169.
9. Tiricz H, et al. (2013) Antimicrobial nodule-specific cysteine-rich peptides induce membrane depolarization-associated changes in the transcriptome of Sinorhizobium meliloti. *Appl Environ Microbiol* 79(21):6737–6746.
10. Boman HG (2003) Antibacterial peptides: Basic facts and emerging concepts. *J Intern Med* 254(3):197–215.
11. Sinharoy S, et al. (2013) The C_2H_2 transcription factor regulator of symbiosome differentiation represses transcription of the secretory pathway gene *VAMP721a* and promotes symbiosome development in *Medicago truncatula*. *The Plant Cell* 25:3584–3601.
12. Maróti G, Kereszt A, Kondorosi E, Mergaert P (2011) Natural roles of antimicrobial peptides in microbes, plants and animals. *Res Microbiol* 162(4):363–374.
13. Kirkpatrick CL, Viollier PH (2011) New(s) to the (Z-)ring. *Curr Opin Microbiol* 14(6):691–697.
14. Kerner MJ, et al. (2005) Proteome-wide analysis of chaperonin-dependent protein folding in *Escherichia coli*. *Cell* 122(2):209–220.
15. Kondorosi E, et al. (1991) Identification of NolR, a negative transacting factor controlling the nod regulon in Rhizobium meliloti. *J Mol Biol* 222(4):885–896.
16. Bittner AN, Foltz A, Oke V (2007) Only one of five groEL genes is required for viability and successful symbiosis in Sinorhizobium meliloti. *J Bacteriol* 189(5):1884–1889.
17. Penterman J, et al. (2014) Host plant peptides elicit a transcriptional response to control the Sinorhizobium meliloti cell cycle during symbiosis. *Proc Natl Acad Sci USA* 111(9):3561–3566.
18. Dzionara M, Kaltschmidt E, Wittmann HG (1970) Ribosomal proteins. 8. Molecular weights of isolated ribosomal proteins of Escherichia coli. *Proc Natl Acad Sci USA* 67(4):1909–1913.
19. Byrgazov K, Vesper O, Moll I (2013) Ribosome heterogeneity: Another level of complexity in bacterial translation regulation. *Curr Opin Microbiol* 16(2):133–139.
20. Ogawa J, Long SR (1995) The Rhizobium meliloti groELc locus is required for regulation of early nod genes by the transcription activator NodD. *Genes Dev* 9(6):714–729.
21. Maunoury N, et al. (2010) Differentiation of symbiotic cells and endosymbionts in Medicago truncatula nodulation are coupled to two transcriptome-switches. *PLoS ONE* 5(3):e9519.
22. Govezensky D, Greener T, Segal G, Zamir A (1991) Involvement of GroEL in nif gene regulation and nitrogenase assembly. *J Bacteriol* 173(20):6339–6346.
23. Login FH, et al. (2011) Antimicrobial peptides keep insect endosymbionts under control. *Science* 334(6054):362–365.
24. Shigenobu S, Stern DL (2013) Aphids evolved novel secreted proteins for symbiosis with bacterial endosymbiont. *Proc Biol Sci* 280(1750):20121952.
25. Futahashi R, et al. (2013) Gene expression in gut symbiotic organ of stinkbug affected by extracellular bacterial symbiont. *PLoS ONE* 8(5):e64557.
26. Kupper M, Gupta SK, Feldhaar H, Gross R (2014) Versatile roles of the chaperonin GroEL in microorganism-insect interactions. *FEMS Microbiol Lett*, 10.1111/1574-6968.12390.
27. McRae D, Miller R, Berndt W (1989) Viability of alfalfa nodule bacteroids isolated by density gradient centrifugation. *Symbiosis* 7:67–80.
28. Boisson-Dernier A, et al. (2001) Agrobacterium rhizogenes-transformed roots of Medicago truncatula for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Mol Plant Microbe Interact* 14(6):695–700.
29. Liang J, Shah DM, Wu YS, Rosenberger CA, Hakimi S (2001) Antifungal polypeptide from alfalfa and methods for controlling plant pathogenic fungi. US Patent 6,916,970.
30. Sessions A, Weigel D, Yanofsky MF (1999) The Arabidopsis thaliana MERISTEM LAYER 1 promoter specifies epidermal expression in meristems and young primordia. *Plant J* 20(2):259–263.