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<td>As Published</td>
<td><a href="http://dx.doi.org/10.1073/PNAS.1610724113">http://dx.doi.org/10.1073/PNAS.1610724113</a></td>
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<tr>
<td>Publisher</td>
<td>National Academy of Sciences (U.S.)</td>
</tr>
<tr>
<td>Version</td>
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<tr>
<td>Accessed</td>
<td>Fri Nov 23 21:13:47 EST 2018</td>
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Disulfide cross-linking influences symbiotic activities of nodule peptide NCR247

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Contributed by Graham C. Walker, July 8, 2016 (sent for review March 11, 2016; reviewed by Sharon R. Long and Baldomero M. Olivera)

Interactions of rhizobia with legumes establish the chronic intracellular infection that underlies symbiosis. Within nodules of inverted repeat-lacking clade (IRLC) legumes, rhizobia differentiate into nitrogen-fixing bacteroids. This terminal differentiation is driven by host nodule-specific cysteine-rich (NCR) peptides that orchestrate the adaptation of free-living bacteria into intracellular residents. *Medicago truncatula* encodes a family of >700 NCR peptides that have conserved cysteine motifs. NCR247 is a cationic peptide with four cysteines that can form two intramolecular disulfide bonds in the oxidized forms. This peptide affects *Sinorhizobium meliloti* transcription, translation, and cell division at low concentrations and is antimicrobial at higher concentrations. By preparing the three possible disulfide-cross-linked NCR247 regioisomers, the reduced peptide, and a variant lacking cysteines, we performed a systematic study of the effects of intramolecular disulfide cross-linking and cysteines on the activities of an NCR peptide. The relative activities of the five NCR247 variants differed strikingly among the various bioassays, suggesting that the NCR peptide-based language used by plants to control the development of their bacterial partners during symbiosis is even greater than previously recognized. These patterns indicate that certain NCR bioactivities require cysteines whereas others do not. The results also suggest that NCR247 may exert some of its effects within the cell envelope whereas other activities occur in the cytoplasm. BacA, a membrane protein that is critical for symbiosis, provides protection against all bactericidal forms of NCR247. Oxidative folding protects NCR247 from degradation by the symbiotically relevant metalloprotease HrP (host range restriction peptidase), suggesting that disulfide bond formation may additionally stabilize NCR peptides during symbiosis.

**Significance**

Terminal differentiation of endocytosed rhizobia into nitrogen-fixing bacteroids inside of legume root nodules is orchestrated by a large family of host-encoded cysteine-rich signaling peptides. These peptides have diverse effects on bacteria, but their molecular mechanisms of action are still unknown. The presence of highly conserved cysteine motifs in this peptide family results in an additional layer of complexity because each individual peptide can potentially form several disulfide-cross-link regioisomers under oxidative conditions. By demonstrating several distinct patterns of relative activities between the three disulfide-cross-linked regioisomers of NCR247, its reduced form, and a variant lacking cysteines, our work suggests that disulfide cross-linking can augment the rich complexity of *Rhizobium*-legume chemical communication required for symbiosis.


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The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1610724113/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1610724113

PNAS | September 6, 2016 | vol. 113 | no. 36 | 10157–10162
(host range restriction peptidase) metalloprotease capable of degrading NCR peptides, symbiotic outcomes are affected at a late developmental stage (12). Although NCR peptides clearly play signaling roles in symbiosis, some members are bactericidal at higher concentrations. The membrane protein BacA plays a critical role in symbiosis by protecting *S. meliloti* cells in the symbiosome from the potential bactericidal action of NCR peptides (13, 14).

Prokaryotes, as well as lower and higher eukaryotes, use peptides as signaling molecules, and the cysteine-rich peptides (CRPs) constitute one of the most important families of these molecules. Particularly well-studied CRPs include conotoxins, neurotoxins produced by cone snails, and defensins, important effectors of innate immunity in vertebrates, invertebrates, insects, and plants (15). Although, *M. truncatula*’s >700 NCR peptides are distinct from its ∼80 defensins (5), the shared structural feature of multiple disulfide bonds is thought to afford a compact structure and resistance to proteolytic and chemical degradation in both peptide families (16). Furthermore, reports indicate that reduced and oxidized forms of some CRP peptides have different biological activities (17–20). In principle, motif-A NCR peptides, which have four cysteine residues, can form three possible disulfide–cross-linking regioisomers whereas motif-B NCR peptides, which have six cysteine residues, can form 15 possible disulfide–cross-linking regioisomers. Nevertheless, there has been no systematic study to compare the biological properties of the various oxidized regioisomers and the reduced isomer on the symbiotic and bactericidal activities of any NCR peptide.

To address the issue of how NCR peptide structure and oxidation state relate to function, we chose the intensively studied NCR247 peptide (Fig. 1). NCR247 is composed of 24 aa, contains four cysteine residues, has a caticonic charge (+5.6) at neutral pH, and also has the highest Boman index (4.6) of any of the NCR peptides (5). NCR247 is expressed in the older cells in the infection zone (zone II), where *S. meliloti* cells stop dividing and start differentiating, and also in interzone II and III, where a sudden growth of bacteroids is visible (21). Gene-expression profiling revealed that each peak was a new species that had an m/z value 4 units less than that of NCR247red. This, and three NCR247red regioisomers formed under these conditions, each of which contains two intramolecular disulfide bonds (Fig. 1C and D).

After HPLC separation and purification of each regioisomer (Figs. S1 and S2), we mapped the disulfide linkages of each peak by assigning the major peak (peak 3) to the NCR247red regioisomer by identifying the peptide fragments generated by extended trypsin digestion (Fig. S3). To distinguish between NCR247red,2-3,4 and NCR247red,2-3,2-4, which yield peptide fragments of identical mass after trypsin digestion, we synthesized the NCR247red,2-3,4 regioisomer by orthogonal protection of cysteines and subsequent pairwise deprotection and oxidation to use as an HPLC standard. Authentic NCR247red,2-3,4 displayed the same HPLC retention time as peak 3, thereby allowing us to infer that peak 1 is the NCR247red,3-2,4 regioisomer (Fig. S1). Our results suggest that NCR247red,2-3,4 is the thermodynamically most stable regioisomer of oxidized NCR247, rather than NCR247red,2-3,2-4, as had been proposed (14). It is possible that NCR247red,2-3,3 may be the most abundant isoform present in *M. truncatula* nodules, but other factors could influence the in vivo distribution of regioisomers. Circular dichroism (CD) spectroscopy did not reveal classical secondary structure features in any of the disulfide–cross-linking regioisomers (Fig. S4).

We also synthesized a derivative of NCR247red in which fluorescein isothiocyanate was conjugated to the N terminus (FITC-NCR247red). This derivative has cell division inhibition and bactericidal activity comparable with unmodified NCR247red (Fig. S5). Because the robust optical signal provided by the fluorophore allows detection of relatively low concentrations of the peptide by HPLC, we used this peptide to evaluate whether NCR247red stays reduced under our in vivo experimental conditions. After incubating...
a sublethal concentration (4 μM) of FITC-NCR247_red aerobically with S. meliloti cells for 3 h, the supernatant was analyzed by HPLC and mass spectrometry. The retention time and mass of the peptide recovered from the supernatant were the same as FITC-NCR247_red (Fig. S6). It is possible that some oxidation occurs within the cells if treated, however, based on data presented below that indicate differences between oxidized and reduced forms in some bioassays, we reason that oxidation does not happen to a great extent.

We then systematically examined the relative abilities of sublethal doses of the three disulfide-linked regioisomers of NCR247, NCR247_red and NSR247 to affect several physiological responses thought to be relevant to symbiosis: (i) the expression of representative ExoS/ChvI- and FeuQ/FeuP-controlled genes and the key regulatory gene ctrA (7), (ii) protein synthesis (21), and (iii) cell division, Z ring formation, and septum formation (3, 7). Additionally, we examined their relative bactericidal activities and resistance to proteolysis.

Effect of NCR247 Disulfide–Cross-Linked Regioisomers on ExoS/ChvI and FeuQ/FeuP Controlled Genes and ctrA. Our previous gene-expression profiling study of a synchronized population of S. meliloti treated with a sublethal concentration (4 μM) of NCR247_1,2,3,4 (7) revealed the up-regulation of two symbiotically important regulons controlled by the ExoS/ChvI and FeuQ/FeuP two-component regulatory systems, respectively. The sensors, ExoS and FeuQ, are histidine kinases that phosphorylate their respective response regulators, ChvI and FeuP. The ExoS/ChvI regulon includes numerous genes required for the synthesis of the symbiotically important exopolysaccharide succinoglycan, as well as the known direct ChvI target smc01581 (24). The FeuQ/FeuP regulon includes ndvA, which encodes an ABC cyclic glucan exporter required for symbiosis (25). In contrast to up-regulation of these two regulons, our transcriptional analysis showed that a sublethal concentration of the NCR peptide decreased the expression of ctrA (7), which encodes a critical cell cycle regulator and master regulator of symbiosis (26).

Using quantitative PCR (qPCR) to compare the expression levels of smc01581 and ndvA genes after treatment of S. meliloti cultures with the NCR247 variants, we found that all disulfide–cross-linked regioisomers significantly up-regulated expression of both genes to similar extents. In contrast, NCR247_red had no effect, and NSR247 had only low activity in up-regulating smc01581 and ndvA expression (Fig. 2 A and B). We observed a different pattern of the relative bioactivities of the five NCR247 peptides when we examined their effects on ctrA expression (Fig. 2 C). All three oxidized NCR247 regioisomers were again equivalently bioactive, reducing ctrA expression to similar degrees, and NSR247 was inactive. However, in contrast to having no effect on the expression of the ExoS/ChvI- and FeuQ/FeuP-regulated genes, NCR247_red was as active as the oxidized peptides in reducing ctrA expression. Taken together, these results reveal that the activity of the reduced isomorph NCR247_red relative to that of the oxidized NCR247 regioisomers depends on the biological attribute being assayed.

NCR247 Regioisomers Selectively Inhibit Bacterial in Vitro Translation. The protein translation machinery is one of the major targets of several signaling peptides in plant–microbe and insect–microbe symbioses (27). Farkas et al. (21) observed that the set of NCR247-interacting proteins identified by affinity chromatography includes ribosomal proteins and subsequently discovered that NCR247_red can inhibit protein synthesis in vitro (21). Consequently, we sought to determine whether the other NCR247 variants inhibit protein synthesis. Guided by the reported assay (21), we used an in vitro translation system in which translation of green fluorescent protein (GFP) mRNA can be monitored by measuring GFP fluorescence. We confirmed that NCR247_red (40 μM) substantially inhibits protein synthesis and observed that the relative abilities of the NCR247 variants to inhibit translation were strikingly different from those we had observed in our transcription assays (Fig. 2 D). NCR247_1,2,3,4 and NCR247_1,3,2,4 inhibited translation, albeit to a lesser degree than NCR247_red. In striking contrast, NCR247_1,4,2,3 was highly effective at inhibiting translation of GFP (Fig. 2 D) and could even interfere with translation at the sublethal level of 4 μM (Fig. S7). Moreover, NSR247 was less effective at inhibiting translation compared to NCR247_red, which indicates that the cysteine residues are generally important but not essential for this activity (Fig. 2 D).

NCR247 Cell Division Inhibition Activity Is Independent of Disulfide Connectivity, and Cysteines Are Not Required. We have previously shown that a sublethal concentration (4 μM) of NCR247_1,2,3,4 does not alter the initiation or progression of DNA replication, such that the cellular DNA content increases from 1C to 2C, for synchronized S. meliloti cultures at the G1 phase. However, NCR247_1,2,3,4 specifically and robustly blocks cell division in S. meliloti in G2 (7). At the sublethal dose of 4 μM, the three NCR247_red regioisomers, NCR247_red, and NSR247 all exhibited similar abilities to prevent the 2C cells from dividing to yield daughter cells with 1C DNA content (Fig. 2 E). Lowering the peptide concentration to 2 μM revealed that one of the minor regioisomers, NCR247_1,3,2,4, was somewhat more active than the other peptides (Fig. 2 F and Fig. S8). We have previously shown that NCR247_1,2,3,4 causes an early block in cell division because very little septation is observed and Z-ring formation is inhibited...
The other two NCR247\textsubscript{ox} regioisomers, NCR247\textsubscript{red} and NSR247, also inhibited the appearance of septa to a similar degree (Fig. S9A). Moreover, when we used an inducible FtsZ-GFP translational fusion protein to visualize the Z-ring in cells in synchronous cultures at the G2 phase (7), we found that each of the five NCR247 variants (4 \mu M) exhibited similar abilities to inhibit Z-ring formation (Fig. S9B).

Taken together, these results show that the pattern of activities of the various NCR peptides in inhibiting cell division differs from the activity patterns obtained from the transcription and translation bioassays. Even though NCR247\textsubscript{1,2-3,4} was the most active at 2 \mu M, the fact that the three disulfide–cross-linked regioisomers had approximately the same activity as NCR247\textsubscript{red} at 4 \mu M indicates that disulfide bond formation is not crucial. Our observation that the NCR247 peptide is active further indicates that none of the cysteines are required for inhibition of cell division, thereby suggesting a different underlying molecular mechanism from those involved in regulation of transcription and translation.

**Disulfide Cross-Linking Influences the Relative Bactericidal Activities of NCR247 Peptides in Wild-Type S. meliloti.** Although both NCR247\textsubscript{red} and NCR247\textsubscript{1-2,3-4} induce bacteroid-like features in free-living *S. meliloti* at sublethal concentrations (3, 7, 14), both peptides reduce the colony-forming ability of *S. meliloti* at higher concentrations (14, 22). We compared the bactericidal activity of the three NCR247 regioisomers (Fig. 3A) and observed that NCR247\textsubscript{1-2,3-4} and NCR247\textsubscript{1-2,3-4} are both \geq 10-fold less active than NCR247\textsubscript{red} and NCR247\textsubscript{1-2,3-4} (22). However, they used a preparation of NCR247\textsubscript{1-3,2-4} that was known to be impure (22), and we now have shown that the impurities in a sample of their peptide are not other NCR247\textsubscript{ox} regioisomers as they had speculated (22) (Fig. S10). Thus, our data indicate that disulfide bonds are not required for NCR247 to restrict bacterial growth against *S. meliloti,* although they can modulate its activity. Of the oxidized species, the most abundant regioisomer, NCR247\textsubscript{1-4,2-3}, has the weakest bactericidal activity against *S. meliloti,* an observation of potential physiological significance. NSR247 has negligible bactericidal activity (Fig. 3A), which indicates that one or more cysteines residues are important for the bactericidal activity of NCR247 against *S. meliloti* even though disulfide bonds do not seem to be required.

**BacA Function Attenuates the Bactericidal Activity of All NCR247 Regioisomers and NCR247\textsubscript{red} Against *S. meliloti.*** The membrane protein BacA is required for *S. meliloti* to establish the chronic intracellular infection that underlies its symbiosis (13) and also for the related mammalian pathogen *Brucella abortus* to establish the chronic intracellular infection that underlies its pathogenesis (28). We found that an *S. meliloti* ΔbacA mutant also displayed increased sensitivity to NCR247\textsubscript{1-3,2-4} and NCR247\textsubscript{1-4,2-3}, as well as NCR247\textsubscript{red}. Thus, our results indicate that the molecular mechanism for BacA-mediated resistance of *S. meliloti* to bactericidal NCR247 peptide works equally well for all conformations of the NCR247 peptide.

As shown for NCR247\textsubscript{1-2,3-4}, the sensitivity of the ΔbacA mutant to killing by all of the NCR247 peptides can be restored to that of a WT strain by expressing a plasmid-borne copy of the *S. meliloti* bacA\textsuperscript{+} gene or its isofunctional *Escherichia coli* ortholog, sbmA\textsuperscript{+} gene (Fig. 3C). Interestingly, despite *E. coli* SbmA being isofunctional with BacA when expressed in *S. meliloti* (29), we observed that an *E. coli* ΔsbmA mutant did not display increased sensitivity to killing by any of the NCR247\textsubscript{ox} regioisomers and was more resistant, rather than more sensitive, to NCR247\textsubscript{red} (Fig. 3B). *E. coli* ΔsbmA mutants have previously been shown to exhibit resistance to killing by several proline-rich peptides. The mechanism of *S. meliloti*’s BacA-mediated resistance to NCR247 will require further investigation and may be complex.

Indeed, BacA/SbmA has been shown to affect both the modification of *S. meliloti* and *B. abortus* lipid A by very long chain fatty acids (VLCFAs) and import of proline-rich peptides. Furthermore, the differing responses of the *S. meliloti* bacA and *E. coli* sbmA mutants to NCR247 peptides indicate that the physiological role of the conserved BacA/SbmA protein in a particular bacterium can also be influenced by other cellular functions. As an example, while BacA is required for *S. meliloti* to establish the nascent bacteroids from the bactericidal property of the NCR247 peptide. The expression of genes encoding functions that could influence the properties and functions of NCR peptides, such as bacterial periplasmic disulfide, oxidoreductases, and glutathione-S-transferases, also varies during the development of the symbiosis (30) (Fig. S11).

**Disulfide Bonding in NCR247 Protects Against Degradation by HrrP.** Although NCR peptides have been proposed to be oxidized in planta (9), our results confirm that NCR247\textsubscript{red} is active in several symbiotically related bioassays. A possible role for disulfide cross-linking of NCR peptides could be to stabilize them against proteolytic degradation (31). Expression of the plasmid-encoded metalloprotease HrrP, which is capable of degrading a range of NCR peptides in vitro, has recently been shown to restrict nodule development and nitrogen fixation of certain plant hosts (12). To examine whether disulfide bond formation modulates the proteolytic stability of NCR247, we performed degradation assays using HrrP and catalytically inactive variant HrrP (E62A). The assays were done using the oxidized and reduced NCR variants as substrates. All three NCR247\textsubscript{ox} regioisomers were markedly less susceptible to degradation by HrrP than NCR247\textsubscript{red} (Fig. 4). At a time point when NCR247\textsubscript{ox} was 80% degraded, NCR247\textsubscript{1-2,3-4} and NCR247\textsubscript{1-4,2-3} exhibited virtually no degradation, and NCR247\textsubscript{1-2,3-4}...
was only 10% degraded. Thus, in addition to the effects that disulfide cross-linking may have on various specific bioactivities of the NCR peptides, our results are consistent with the disulfide bonds also providing resistance to proteolytic degradation during the development of the symbiosis.

**Discussion**

In this work, we report a systematic investigation of how disulfide bond formation affects the biological activities of an NCR peptide. Using chemical synthesis, we prepared the three possible oxidized regioisomers of *M. truncatula* NCR247 and compared the activities of these peptides with NCR247_red and NSR247 in a variety of bioassays related to symbiosis. Our studies revealed that the relative activities of these NCR247 variants are strikingly different depending on the bioassay. More than 700 NCR peptides have been identified to date, and this work indicates that disulfide cross-linking provides an additional source of structural and functional diversity. Thus, the subtleties and complexities of how plant NCR peptides manipulate and fine-tune activities of their endosymbiotic partner may be even greater than previously recognized. Our observations are consistent with a growing body of evidence that the disulfide–cross-linked and reduced forms of defensins can have different biological activities (20). Our results also suggest that disulfide cross-linking may be important because it protects the NCR peptides from degradation by host or bacterial proteases during symbiosis.

Several distinct patterns of relative activities of the NCR247 variants for symbiotically relevant functions are evident in our data. One pattern is seen for genes controlled by the ExoS/ChvI and FeuQ/FeuP two-component regulatory systems. The three NCR247_red regioisomers exhibit similar inducing activity whereas NCR247_red and NSR247 are inactive or have only weak activities. Both the ExoS and FeuQ sensors span the membrane and have a periplasmic sensing domain and a cytoplasmic domain that activates their cognate response regulator. One possible explanation for our results is that disulfide cross-linking protects the peptide from proteolytic degradation from bacterial outer membrane, periplasmic, or membrane proteases, so that it can act in the periplasm or membrane to activate the sensors, whereas the non-cross-linked forms are either rapidly degraded in the periplasm or membrane or imported into the cytoplasm. Another possibility is that the oxidized peptides affect the cellular redox balance in a manner that can be detected directly or indirectly by the ExoS/ChvI and FeuQ/FeuP two-component regulatory systems. The similar activities of the three regioisomers suggest that they do not act by binding to one special site on a regulatory protein. However, we cannot rule out the possibility of a rapid interconversion between the regioisomers catalyzed by a periplasmic disulfide isomerase, with one particular regioisomer being capable of such binding.

A second pattern was seen with respect to the reduction of *ctr4* expression, with all three NCR247_red regioisomers and NCR247_red being approximately equally effective, but NSR247 being inactive. Because many of the key upstream regulators of *ctr4* expression are cytoplasmic proteins (26, 32), these observations are consistent with models in which the effects of NCR247 are exerted after it has entered the cytoplasm (21) and the disulfide–cross-linked regioisomers become reduced. Nevertheless, our data suggest that at least one cysteine of NCR247 is required for *ctr4* regulation.

A third pattern was observed in the assays for inhibition of cell division, Z-ring formation, and septation, with all five NCR247 variants exhibiting similar activity. Because the oxidized forms of NCR peptides are likely to be reduced upon entry into the cytoplasm, this pattern is consistent with the reduced form acting inside the cell. Notably, unlike *ctr4* regulation, there is no apparent requirement for the NCR247 peptide to contain a cysteine for these activities. These observations are consistent with the suggestion by Farkas et al. (21) that NCR247 may exert some of its effects by interacting with intracellular proteins such as FtsZ. The high Boman index of NCR247 is consistent with the evidence that it is capable of interactions with multiple partner proteins (21).

A fourth pattern is evident in the case of inhibition of *in vitro* translation: NCR247_red is a potent inhibitor, NCR247_red and NSR247 are less active inhibitors, and the other two regioisomers are very weak inhibitors. These data suggest that NCR247_red binds to a specific target in the translational apparatus that is poorly bound by the other two regioisomers because of their differing 3D shapes and that NCR247_red and NSR247 may be able to adopt conformations similar to that of NCR247_red when they bind to this target. As noted, one would expect oxidized forms of NCR247 to be converted to NCR247_red upon entry into the cytoplasm. However, rhizobia are subjected to oxidative stress while establishing the symbiosis (33), and prolonged exposure to NCR247 might possibly lead to oxidative stress, as has been reported for certain antimicrobial peptides. Bassette et al. (34) have shown that the bacteria can be made sufficiently oxidizing to allow the formation of native disulfide bonds without compromising cell viability.

The pattern of the relative activities of the NCR247 variants for bactericidal action has some resemblance to that for *ctr4* regulation, in that all three regioisomers and NCR247_red are active whereas NSR247 is inactive. However, in this case, NCR247_red, red, and NCR247_red, 3,4 are both at least ten times less active than NCR247_red and NCR247_red, 3,4. Thus, not only does the specific nature of the disulfide cross-linking influence the bactericidal potency of the NCR247 regioisomer, but also the NCR247 peptide must contain one or more cysteines to kill cells even when it is reduced.

The exquisite potency and selectivity that conotoxins display for a range of ion channels and receptors illustrates the high degree of biological specificity that can be achieved by cysteine-rich peptides. NCR peptides have been shown to be undergoing rapid and recent evolution, which suggests that the family of NCR genes is rapidly changing to adapt to different environments and to acquire new functions (35). Nevertheless, the conservation of the 4- and 6-cysteine motifs indicates their importance for NCR function. Horváth et al. (10) have shown that replacement of any of the four cysteines in *Ncr169* prevents it from carrying out its role in symbiosis. However, it is not yet clear whether this observation is due to an effect on peptide stability, peptide signaling functions, or both.

Antimicrobial peptides are an ancient component of the innate immune system whereas the legume–Rhizobium symbiosis arose only about 60 million years ago. Furthermore, the involvement of NCR peptides in the host–microbe interaction is thought to have been a relatively recent innovation within a subclass of legumes (35) that may lead to more efficient nitrogen fixation or to increased release of rhizobia into the environment after senescence (5). Thus, the bactericidal activity that some of the NCR peptides exhibit at relatively high concentrations may simply reflect their evolutionary relationship to defensins (35, 36). Nevertheless, it is also possible that this activity may help the plant to maintain an internal monoclone by killing any...
susceptible bacterium that manages to coin evade along with the rhizobial symbiont. Along these lines, the BacA protein plays a critical role in symbiosis by preventing the rhizobia from being killed by the concentrations of NCR peptides present in the symbiosome, so that the signaling roles of the NCR peptides can predominate.

NCR peptides affect multiple components of bacterial physiology to achieve a successful symbiosis, some inducing the final terminal differentiated state and others affecting bacteroid persistence. Some NCR peptides may work cooperatively with other NCR peptides to exert positive or negative effects on affected pathways whereas multiple NCR peptides may bind to the same target to achieve their ultimate biological effects. Although some NCR-peptide interactions are well-characterized, the two NCR-peptide family members have unique functions essential for symbiosis (NCR169 and NCR211) (10, 11). Which physiological functions require peptide interactions with membranes and which may require interactions with specific cellular proteins remain to be clarified. Cationic peptides can disrupt cellular membranes, consequently some cationic NCR peptides may affect membrane permeability, leading to physiological outcomes and also permitting other classes of NCR peptides to enter the bacteria. Interactions of an NCR peptide with a specific protein target would probably be strongly affected by the pattern of disulﬁde cross-linking.

Materials and Methods

Detailed materials and methods can be found in SI Materials and Methods.

Bacterial Growth Conditions. For all experiments, S. meliloti 1021 WT and ΔsbmA-null mutant strains were grown in Lysogeny broth (LB) prepared with 1% (wt/vol) Bacto-Tryptone and supplemented with 2.5 mM CaCl2 and 2.5 mM MgSO4 (LB- MC) for 48 h at 30 °C. E. coli WT and ΔzmdA-null mutant strains were grown in LB (5 g·L−1 NaCl) at 37 °C. Unless indicated otherwise, S. meliloti cultures were supplemented with 200 μg·mL−1 streptomycin (5μm).

Foldin and Disulﬁde Formation. The folding reaction typically proceeded at room temperature overnight in a sealed vial with gentle stirring. Oxidation of NCR247_red was done in 20% DMSO in water (23, 37).

ACKNOWLEDGMENTS. We thank all members of the G.C.W. and E.M.N. laboratories and Caroline Koehler for helpful discussions. We thank Ms. Deborah Pheasant (Biophysical Instrumentation Facility) for help in the CD spectroscopy experiments and the BioMicro Center for RT-qPCR facilities. This work was supported by National Institutes of Health (NIH) Grant GM31010 (to G.C.W.), NIH Grant 1DP2OD007045 (to E.M.N.), NIH Grant P30 ES002109 to the MIT Center for Environmental Health Sciences, and a Human Frontier Science Fellowship (to M.S.). J.G. and P.A.P. were supported by National Science Foundation Grant IOS-1054980 and J.S.G. was also supported by USDA/National Institute of Food and Agriculture Grant 2015-67013-22915. H.T.B. was supported by the Elisabeth Meurer Foundation. G.C.W. is an American Cancer Society Professor.