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Disulfide cross-linking influences symbiotic activities of nodule peptide NCR247

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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. BagA, 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-linked 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.
(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-linked regioisomers whereas motif-B NCR peptides, which have six cysteine residues, can form 15 possible disulfide–cross-linked regioisomers. Nevertheless, there has been no systematic study of the regioisomers of the various oxidized regioisomers and the reduced isoform 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 cationic charge (+5.6 e) at neutral pH, and also has the highest Roman 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 treatment of a synchronized S. meliloti population with a sublethal level of NCR247 affects ~15% of the genome, including three symbiotically important regulons (7). Two of these regulons are controlled by the ExoS/ChvI and FeuQ/FeuP two-component systems, respectively, and the other by the master cell cycle regulator ctgA. Sublethal levels of NCR247 permit the cell to complete one round of DNA replication but then lead to inhibition of cell division, Z-ring formation, and septation (7). NCR247 also inhibits protein synthesis (21). Potential interacting partners of NCR247 by using affinity chromatography include FtsZ, numerous ribosomal proteins, the GroEL chaperone, and RNA polymerase units (21). Both the reduced isoform of NCR247 (NCR247red) and one disulfide-linked NCR247 regioisomer have been shown to be bactericidal at higher concentrations (3, 22).

Our results demonstrate that the particular disulfide–cross-linked regioisomers of NCR peptides and the reduced NCR peptides can differ strikingly in their abilities to cause various biological effects. Taken together, these observations suggest that the NCR peptide-based language that plants use to control the development of their bacterial partner during symbiosis may be more complex than currently appreciated.

Results

Preparation of the Three Disulfide-Linked Regioisomers of NCR247.

Previous studies of the biological activities of NCR247 have used the reduced isoform NCR247red (3, 22) or the disulfide-linked NCR2471-2,3-4 regioisomer (7, 14) (Fig. 1A). The latter form has been proposed (22) to be the isoform present in root nodules on the basis of Chou–Fasman predictions (14). To gain insights into the potential importance of disulfide bonds and cysteines to the biological activities of NCR247, we prepared the three disulfide-linked regioisomers of NCR247 (Fig. 1A). We then compared the biological activities of these oxidized peptides with those of NCR247red and also of NSR247 (22). NSR247 is an NCR247 variant that cannot form disulfide bridges because all four cysteine residues are replaced by serines.

We obtained NCR247red by Fmoc solid-phase peptide synthesis and used DMSO in oxidative folding assays. This mild oxidant has been used to fold basic and hydrophobic peptides, including human and mouse prepro-α-defensin 3 (23). We obtained NCR247red in 20% (vol/vol) DMSO solution (room temperature for 20 h) resulted in the formation of three species, which could be separated by HPLC (high-performance liquid chromatography) (Fig. 1B). Mass spectrometry revealed that each peak was a new species that had an m/z value 4 units less than that of NCR247red. Thus, all 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 NCR2471-2,3-4 regioisomer by identifying the peptide fragments generated by extended trypsin digestion (Fig. S3). To distinguish between NCR2471-2,3-4 and NCR2471-3,2-4, which yield peptide fragments of identical mass after trypsin digestion, we synthesized the NCR2471-2,3-4 regioisomer by orthogonal protection of cysteines and subsequent pairwise deprotection and oxidation to use as an HPLC standard. Authentic NCR2471-2,3-4 displayed the same HPLC retention time as peak 3, thereby allowing us to infer that peak 1 is the NCR2471-3,2-4 regioisomer (Fig. S1). Our results suggest that NCR2471-2,3-4 is the thermodynamically most stable regioisomer of oxidized NCR247, rather than NCR2471-2,3-4, as had been proposed (14). It is possible that NCR2471-4,2-3 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-linked 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

![Fig. 1](https://www.pnas.org/ cgi/doi/10.1073/pnas.1610724113)

**Fig. 1.** Generation and analyses of NCR247 regioisomers. (A) Primary sequences and illustration of disulfide bond linkages in NCR247red. The brackets indicate the disulfide bond linkages in the oxidized peptides. (B) HPLC trace (10-60% acetonitrile over 30 min, 1 mL/min, 280 nm) showing that oxidation of NCR247red in 20% DMSO generated three new peaks, which are assigned as the NCR247red regioisomers. (C and D) Deconvoluted ESI-MS spectra of NCR247red and NCR247red variants indicate the oxidation state of the peptides.
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 occurred within the cells of S. meliloti, 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 regiosomers 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 Regiosomers 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_red (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 regiosomers 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. 2A 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. 2C). All three oxidized NCR247 regiosomers 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 isomeric NCR247_red relative to that of the oxidized NCR247 regiosomers depends on the biological attribute being assayed.

**NCR247 Regiosomers 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. 2D). NCR247_red inhibited translation, albeit to a lesser degree than NCR247_red. In striking contrast, NCR247_red was highly effective at inhibiting the translation of GFP (Fig. 2D) 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. 2D).

**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_red 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_red specifically and robustly blocks cell division in S. meliloti in G2 (7). At the sublethal dose of 4 μM, the three NCR247_red regiosomers, NCR247_red, and NSR247 all exhibited similar abilities to prevent the 2C cells from dividing to yield daughter cells with 1C DNA content (Fig. 2E). Lowering the peptide concentration to 2 μM revealed that one of the minor regiosomers, NCR247_red, was somewhat more active than the other peptides (Fig. 2F and Fig. S8). We have previously shown that NCR247_red causes an early block in cell division because very little septation is observed and Z-ring formation is inhibited.

![Fig. 2. Treatment with NCR247 variants affects expression levels of genes regulated by the ExoS/ChvI, FeuQ/FeuP regulons and of the master cell-cycle regulator CtrA. (A and B) Fold change in smc01581 (A) and ndvA (B) expression relative to smc00128 after 15 min with no peptide or with a sublethal concentration (4 μM) of peptide-treated synchronized cultures. (C) The ctrA expression in nontreated and peptide-treated synchronized cultures relative to smc00128. (D) NCR247_red and NCR247_red (4 μM) specifically inhibit protein synthesis in vitro. (D, Upper) NCR247 variants (40 μM) were added to the “in vitro translation reaction” mixture, and GFP fluorescence was measured by using a fluorescence plate reader after 3 h (λex = 485 nm, λem = 530 nm). (D, Lower) Gel fluorescence of GFP reporter protein after 3 h of treatment with peptide. (E and F) DNA content per cell during synchronized growth of S. meliloti cultures treated with or without 4 μM (E) and 2 μM (F) of NCR247 variants. DNA content per cell during synchronized growth of S. meliloti cultures treated with or without NCR247 variants. Flow cytometry was used to measure the percentage of cells with one genome or two genomes. All error bars represent SD from the mean, based on triplicate experiments.](image)
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 Ptz-Z-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 \textmu 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 \textmu M, the fact that the three disulfide–cross-linked regioisomers had approximately the same activity as NCR247\textsubscript{red} at 4 \textmu 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{1-3,2-4} are both \textgeq 10-fold less active than NCR247\textsubscript{red} and NCR247\textsubscript{1-3,2-4} (22). In this respect, our results for NCR247\textsubscript{1-2,3-4} differ from those of Haag et al. (22), who reported that NCR247\textsubscript{1-3,2-4} is less bactericidal than NCR247\textsubscript{red} (22). However, they used a preparation of NCR247\textsubscript{1-2,3-4} 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 be bactericidal, 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 cysteine 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 pathogenesis (28). We found that an S. meliloti \Delta bacA 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.

Although both BacA and SbmA are membrane proteins, the sensitivity for NCR247\textsubscript{1-2,3-4}, the sensitivity of the \Delta sbmA 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 iso-functional 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 \Delta sbmA 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. S8B). E. coli \Delta sbmA 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 discussed previously (29), the expression of NCR247 and two other intensively studied NCR peptides (NCR211 and NCR169) roughly coincides with bacA, consistent with BacA protecting 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{red} was 80% degraded, NCR247\textsubscript{1-3,2-4} 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_{ox} 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 *ctrA* expression, with all three NCR247_{ox} regioisomers and NCR247_{red} being approximately equally effective, but NSR247 being inactive. Because many of the key upstream regulators of *ctrA* 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 *ctrA* 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 *ctrA* 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_{1,4,2,3} 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_{1,4,2,3} 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_{1,4,2,3} when they bind to this target. As noted, one would expect oxidized forms of NCR247 to be converted to NCR247_{ox} 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. Bessette et al. (34) have shown that the bacterial cytoplasm 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 *ctrA* regulation, in that all three regioisomers and NCR247_{red} are active whereas NSR247 is inactive. However, in this case, NCR247_{1,4,2,3} and NCR247_{1,2,3,4} are both at least ten times less active than NCR247_{red} and NCR247_{1,2,3,4}. Thus, not only does the specific nature of the disulfide cross-linking determine 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 defense against chemical defense mechanisms. These observations suggest that the NCR247 variants 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 monoculture by killing any
suscetible bacterium that manages to coevolve 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 symbiotic zone, 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 peptides do not interact with bacterial cell membranes, NCR peptides are found to bind with high affinity to multiple regions of at least two unique functionally 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 ΔbacA-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 MgSO₄·7H₂O (LB- MC) for 48 h at 30 °C. E. coli WT and ΔзнаА-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 μM L- streptomycin (5 μM).

Folding and Disulfide Formation. The folding reaction typically proceeded at room temperature overnight in a sealed vial with gentle stirring. Oxidation of NCR247red was done in 20% DMSO in water (23, 37).

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5. Maróti G, Downie JA, Kondorosi É (2015) Plant cysteine-rich anti-microbial peptides th...