



Why Should Nodule Cysteine-Rich (NCR) Peptides Be Absent From Nodules of Some Groups of Legumes but Essential for Symbiotic N-Fixation in Others?

J. Allan Downie^{1*} and Eva Kondorosi²

¹ Department of Molecular Microbiology, John Innes Centre, Norwich, United Kingdom, ² Institute of Plant Biology, Biological Research Center of the Eötvös Loránd Research Network, Szeged, Hungary

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*Correspondence:

J. Allan Downie
allan.downie@jic.ac.uk

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In nitrogen-fixing nodules of legumes such as pea (*Pisum*) and *Medicago* spp. the plant induces terminal differentiation in the rhizobial endosymbionts by targeting nodule-specific cysteine-rich defensin-like peptides into the bacteria. However, in nodules of other legumes such as soybean and *Lotus* spp. terminal bacterial differentiation does not occur; these legumes lack genes encoding equivalent peptides controlling rhizobial development. Here, we review the effects of some of these peptides on rhizobia and address the question as to how and why such peptides may have evolved to enslave rhizobia and become essential for nitrogen fixation in some clades of legumes but not in others.

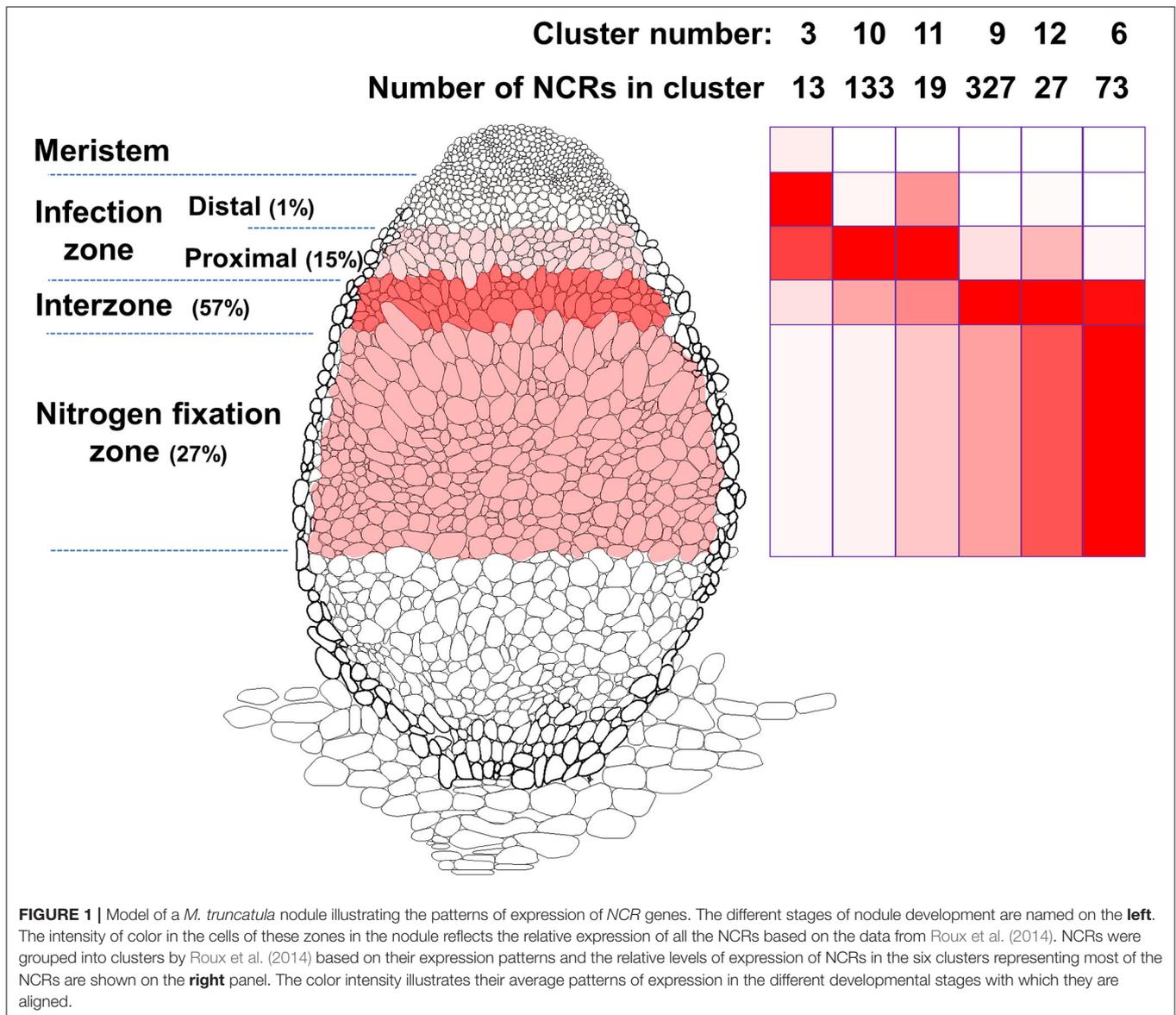
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INTRODUCTION

About 90% of 19,000 species of legumes have nitrogen-fixing nodules, which can have diverse structures in different legume genera (Sprent, 2001). There is a profound difference in the fate of nitrogen-fixing rhizobia in different types of legume nodules because in some, the rhizobia become terminally differentiated and cannot be cultured, whereas in others, the nitrogen-fixing rhizobia can return to the free-living state. These differences are caused by plant-encoded small peptides that can induce terminal bacterial differentiation.

Symbiotic nitrogen fixation has been most studied in legumes producing root nodules with either “determinate” or “indeterminate” meristems, that are usually infected by rhizobia entering roots *via* plant-made infection threads formed after rhizobial-legume signaling (Oldroyd et al., 2011). The infection threads are tunnel-like structures that allow the rhizobia to grow into the region of the root in which cell proliferation has initiated nodule organogenesis (Oldroyd et al., 2011).

“Indeterminate” nodules are cylindrical with a continuously active meristem, a bit like very stubby lateral roots. Such nodules, which e.g., are formed on *Pisum*, *Medicago* spp., have an age gradient along their length with a growing meristem at the tip, an infection zone, a nitrogen-fixation zone and a so-called interzone between the infection and nitrogen-fixation zones (**Figure 1**). Rapid bacterial differentiation occurs in the interzone and is initiated in the proximal infection zone after the bacteria are released from infection threads and are endocytosed into the cytoplasm surrounded by a plant-made membrane. Many of the legumes forming indeterminate nodules lack an ancient



inverted repeat region indicating a deep phylogenetic split with other legumes (Wojciechowski et al., 2004); consequently, they are referred to as the “Inverted-Repeat-Lacking Clade” (IRLC) of legumes.

“Determinate” nodules such as those formed on soybean, *Phaseolus* bean and *Lotus* spp. are spherical and develop as a consequence of transient cell proliferation; so all infected cells in mature nodules are essentially at the same developmental stage.

NCR PEPTIDES

Peptides Control Rhizobial Development in Some but Not Other Legumes

In the nodules of IRLC clade legumes such as pea and *Medicago* spp., nitrogen-fixing bacteria (bacteroids) become terminally

differentiated and cannot be cultured (Mergaert et al., 2006), whereas with legumes like soybean and *L. japonicus*, nitrogen-fixing rhizobia do not terminally differentiate and can regrow (Gresshoff and Rolfe, 1978). Terminally differentiated nitrogen-fixing bacteroids show chromosomal endoreduplication, arrest of cell division, cell enlargement and changes in cell walls, membrane permeability, and patterns of gene expression (Batut et al., 2011; Kereszt et al., 2011; Haag et al., 2013; Kondorosi et al., 2013; Maroti and Kondorosi, 2014; Alunni and Gourion, 2016). Many of these changes are caused by plant-made small defensin-like peptides. This has been best characterized in *M. truncatula* in which there are two gene families, one (of about 650) encoding Nodule-specific Cysteine-Rich (NCR) peptides and one (of 24) encoding Glycine-Rich Peptides (GRPs). Similar genes have been identified in other members of the IRLC legumes (Kevei et al.,

2002; Graham et al., 2004; Alunni et al., 2007; Montiel et al., 2017) but neither NCR nor GRP-encoding genes could be identified in the sequenced genomes of *L. japonicus* and soybean (Alunni et al., 2007). The NCRs are delivered through the plant-made membrane surrounding the bacteroids *via* a plant-determined secretion complex that recognizes the highly conserved N-terminal secretion signal on NCR peptides (Wang et al., 2010; Stonoha-Arther and Wang, 2018). The mature peptides (usually 35–55 residues) then enter the bacteroids, inducing the changes associated with terminal differentiation (Mergaert et al., 2006; Van de Velde et al., 2010; Tiricz et al., 2013; Farkas et al., 2014; Penterman et al., 2014). The effects of NCR peptides have been reviewed recently (Mergaert et al., 2020; Roy et al., 2020).

NCR Peptides: Activity and Rhizobial Protection Mechanisms

Most eukaryotes produce anti-microbial defensin peptides and these fall into different groups including cysteine-rich peptides that bind to microbial proteins. In such defensins, cysteine crosslinks hold together α -helical and β sheet regions such that highly variable “interactive” domains can bind efficiently to proteins (Shafee et al., 2017). NCRs are similar to, but different from most cysteine-rich defensins, usually having 4 or 6 cysteine residues rather than 8 or 10 seen in true defensins (Maroti et al., 2015). The sequences of NCR peptides are highly diverse and fall into cationic, anionic, and neutral groupings. The NMR-derived structure of NCR044 produced in *Pichia pastoris* revealed that it was mostly disordered, highly dynamic and internally cross-linked via *two* disulfide bonds that linked two antiparallel beta strands and linked one of these to a very short potential alpha helix. NCR044 entered cells of the fungal pathogen *Botrytis cinerea* via discrete membrane foci causing a loss of turgor and the production of reactive oxygen species (Velivelli et al., 2020). Another peptide (NCR247) can bind to multiple proteins in bacteroids (Farkas et al., 2014) and this is associated with inhibition of transcription, translation and cell division (Farkas et al., 2014; Penterman et al., 2014; Shabab et al., 2016). Cysteine crosslinking within NCR247 was essential for effects on transcription, was important but not essential for inhibition of translation and was not required for inhibition of cell division (Haag et al., 2011, 2012; Shabab et al., 2016). Rhizobia require some degree of protection against such potentially widespread disruptive effects. Some *S. meliloti* strains have a plasmid-encoded peptidase (HrrP), that suppresses nitrogen fixation on some *Medicago* species but not on others (Crook et al., 2012; Price et al., 2015). HrrP can degrade several NCR peptides resulting in premature nodule senescence and a defective symbiosis, similar to the phenotypes caused by mutations in *NCR* genes (see below). Another mechanism conferring protection against NCRs is mediated via rhizobial *bacA* (or *bclA*) genes that are essential for terminal bacteroid development in IRLC legumes but not in legumes in which nitrogen-fixing rhizobia can regrow (Glazebrook et al., 1993; Karunakaran et al., 2010; Maunoury et al., 2010; Guefrachi et al., 2015; Barriere et al., 2017). *BacA* may play a role in the transport of peptides, and the *bacA* mutation increased sensitivity to the

stable folded form of NCR247 (Haag et al., 2011). *BacA* and *BclA* have been implied to act either by importing NCR peptides to remove them from the bacterial cell surface (a likely site of activity), or by exporting them to reduce their cytoplasmic toxicity (Haag et al., 2011, 2013; Barriere et al., 2017). Mutations affecting rhizobial cell envelope polysaccharides, inner and outer membrane proteins and transcriptional regulators affect the sensitivity to NCR247 (Arnold et al., 2017, 2018).

Different Legumes Have Widely Differing Numbers of NCR Peptides

M. truncatula has >650 NCR peptides predicted from genome sequencing (Young et al., 2011) of which about 600 were identified in RNA sequencing of nodule tissue (Roux et al., 2014). The expression of NCR peptides in nodules was reduced by added nitrate (Liese et al., 2017; Schulze et al., 2020) in parallel with nitrate-induced decreases in other nodule proteins. Mutations in specific NCR genes (Horvath et al., 2015; Kim et al., 2015), or affecting the secretion complex that delivers the peptides to bacteroids, abolish symbiotic nitrogen fixation (Van de Velde et al., 2010; Wang et al., 2010).

IRLC legumes express different numbers of NCRs; 7 were identified in *Glycyrrhiza uralensis* (Chinese licorice), 63 in *Cicer arietinum* (chickpea), 353 in *Pisum sativum* (pea), and 469 in *M. sativa* (alfalfa). The degree of bacteroid differentiation in the tested legumes correlated with the number and composition of NCR peptides (Montiel et al., 2016, 2017). The ability of the legumes to induce bacteroid swelling (an aspect of differentiation) was predicted to be acquired independently in at least five independent lineages and bacteroid morphotypes even within a single legume can vary (Oono et al., 2010). Such differentiation is not necessarily imposed on all rhizobia nodulating legumes in the IRLC clade. For example, in *G. uralensis* nodules *Sinorhizobium fredii* strain HH103 did not show the signs of bacteroid differentiation (Crespo-Rivas et al., 2016) that were seen with *Mesorhizobium tianenshenense* isolated from *G. uralensis* nodules (Montiel et al., 2016, 2017). This fits with the observation that *S. fredii* HH103 showed little *in-vitro* sensitivity to NCR peptides (Crespo-Rivas et al., 2016), that are toxic to other rhizobia (Tiricz et al., 2013). However, only cationic NCR peptides with a pI > 9.5 have so far been confirmed to have antimicrobial activity (Van de Velde et al., 2010; Ordogh et al., 2014) and *G. uralensis* lacks such NCRs.

Phylogenetic analyses indicated there were many legume-species-specific NCRs (Montiel et al., 2017). Such independent evolution of NCR peptides is consistent with the conclusion that there has been convergent evolution of endosymbiont differentiation driven by NCR-like peptides in the Dalbergoid clade of legumes that evolved separately from the IRLC clade (Czernic et al., 2015). However, the diversity in number and sequences of NCR peptides also point toward rapid evolution and diversification of this group of genes; the chromosomal clustering of groups of NCR genes and pseudogenes with related sequences in *M. truncatula* (Young et al., 2011) indicates that this diversification occurs *via* gene duplication. A comparison of 26 mature NCR sequences from different accessions of *M.*

truncatula revealed a relatively high pattern of diversifying selection consistent with recent and rapid evolution leading to new functions of NCRs (Nallu et al., 2013). A comparison of NCR peptides in pea and lentil also indicated parallel evolution of NCR peptides and NCR genes arising from gene duplication (Duran et al., 2021). These observations imply that a single rhizobial genotype can encounter different NCR peptides in nodules within the same cross-inoculation group of legumes.

NCR Genes Regulate Bacteroid Development and Rhizobial Strain Discrimination

The rapid evolution and selection of NCRs implies that they confer a strong selective advantage. However, the absence of NCR peptides in several nitrogen-fixing legumes shows that NCR peptides are not required *per se* for symbiotic nitrogen fixation. Therefore, IRLC legumes probably use NCRs to manipulate rhizobia to optimize nitrogen fixation. The distribution of NCRs and the effects of mutations in individual NCR genes raise a few paradoxes. Firstly, NCRs have antimicrobial activity *in vitro* and yet the loss of individual NCR genes causes rapid senescence of bacteroids (Horvath et al., 2015; Kim et al., 2015). Secondly, although mutations in individual NCRs such as NCR169 and NCR211 cause rapid bacteroid senescence, these NCRs are not required for nitrogen fixation in those legumes lacking all NCRs. Thirdly, why should some legumes such as *G. uralensis* manage with few (Montiel et al., 2017) when *M. truncatula* has >600 nodule-expressed NCR genes?

One reason for having different NCR genes could relate to their expression pattern. **Figure 1** was made based on RNA sequencing and identification of gene expression clusters of NCR genes (Roux et al., 2014). It shows the pattern of expression of about 600 NCRs in different tissues of *M. truncatula* nodules; analysis of these data reveals that cationic NCR peptides with antimicrobial activity are restricted to the interzone and nitrogen fixing zone. Few (if any) NCR genes are expressed in the nodule meristem. Some NCRs are expressed in the distal part of the infection zone, in which many rhizobia remain in infection threads or some have recently been endocytosed into plant cells. Most rhizobia are released from infection threads in the proximal infection zone, where about 15% of the nodule-expressed NCR genes are induced. Most (56%) NCR genes are expressed in the interzone, which corresponds to the region of the nodule in which *S. meliloti* bacteroids are differentiating. Thus, over 70% of the NCR genes are induced before the onset of nitrogen fixation and many of these genes are switched off in the nitrogen fixation zone. The transient pattern of transcription of many NCR genes is correlated with the increasing ploidy levels of both the differentiating rhizobia (Mergaert et al., 2006) and the plant cells as they differentiate (Nagyimihaly et al., 2017).

Different NCRs may enable legumes to discriminate against some rhizobia. Rhizobial-specific nitrogen fixation appears to be common among different accessions of *M. truncatula* (Liu et al., 2014) and discrimination between some rhizobial strains can be attributed directly to the presence of specific NCR genes (Wang et al., 2017, 2018; Yang et al., 2017). As observed in alfalfa and *M.*

truncatula, different NCRs can be induced by different rhizobial strains (Burghardt et al., 2017; Kang et al., 2020). Mutation of some NCR genes conferred nitrogen fixation on a strain that was unable to fix nitrogen in plants carrying the NCR gene. One of these NCR genes was expressed in the proximal infection and transitional zones (Yang et al., 2017). This is an odd stage to apply sanctions to some rhizobia, because it follows the commitment to nodule development, but precedes an assessment of effectiveness of nitrogen fixation. Perhaps the effects of some NCRs could be an undesirable consequence of NCR function. A focus of future research will be to identify the modes of action of different NCR peptides.

DISCUSSION

Why Are There So Many NCRs in Some Legumes?

To address this, we should consider the potential roles of NCR peptides. Some cationic peptides cause membrane depolarization and disintegration *in vitro* (Tiricz et al., 2013). This may be due to the relatively high concentrations used; at low concentrations labeled NCR247 could enter cells without causing membrane damage but at higher concentrations increased membrane permeability. It seems unlikely that *M. truncatula* would produce >600 proteins primarily causing membrane permeabilization and so these effects are probably not the primary role of most NCR peptides. Tagged NCR247 (Farkas et al., 2014) pulled down proteins associated with at least nine separate complexes (Ribosomal proteins, FtsZ, GroEL, Pyruvate dehydrogenase, transaldolase, RNA polymerase, Elongation factors, a Maf-like protein, and nitrogenase). Thus, some NCRs, like antimicrobial peptides, are probably “sticky” and can bind to many other proteins, possibly inhibiting or modulating their function. If even only 10% of the 600 or so NCRs expressed in *M. truncatula* nodules are “sticky,” then these NCRs have the potential to modulate activity of over a 100 rhizobial proteins. Why should some legumes evolve so many NCR peptides and to what end? Three reasons for having NCRs could be: (1) The NCRs may constrain rhizobial growth in nodules. (2) The NCRs may play a role in selection against cheaters (non-nitrogen fixing bacteria that get into nodules) or to select against rhizobia with poor nitrogen fixation. (3) Plants may use NCRs to take control of bacteroid development (and possibly aspects of metabolism) to optimize nitrogen fixation. Since each would induce positive selection, it is probable that some legumes combine aspects of each.

How might this have evolved? How can we explain why the loss of a single NCR can block nitrogen fixation, whereas other legumes fix nitrogen without that specific NCR? Let us assume that an early role of NCRs was to suppress growth in nodules of cheaters (or rhizobia that are poor at N-fixation). A nodule-expressed NCR may have evolved from a defensin to suppress growth of a cheater by targeting a protein that is absent from (or sufficiently different from that of) the rhizobial endosymbiont. This could confer symbiotic benefit and

subsequent gene duplication and diversification could allow the acquisition of several related NCRs targeting different cheaters. However, the observation that a single NCR can bind to several targets implies that some side effects might negatively affect the symbiosis, for example by causing accumulation of some intermediate that limits symbiotic nitrogen fixation. If this did occur, the plant could resolve the problem by losing the NCR peptide. Alternatively, it could in theory, suppress the negative side effects by acquiring another NCR peptide that could down-regulate more of the pathway thereby decreasing accumulation of the problematic intermediate. It is possible that the acquisition of several NCRs could enable the plant to control different aspects of rhizobial development (and possibly metabolism) such that there would be selection against cheaters and optimization of nitrogen fixation. However, once such a control network had developed it could easily be associated with an interdependence of NCRs, such that e.g., if the first NCR was lost (e.g., by mutation) then an imbalance in the control network due to its loss, could result in a poor or ineffective symbiosis as seen with loss of NCR 169 or NCR211. Acquisition of NCRs that benefit some rhizobial strains could be detrimental to the symbiosis established with others. Therefore, there is the potential for both positive and negative selection, which could explain the observed diversifying selection (Nallu et al., 2013).

Why Are NCR Genes Absent From Many Legumes?

If the acquisition of NCRs is of some benefit to legumes in the IRLC species, why are they not present in legumes such as soybean and *Lotus* spp? One possibility is that there may be a disadvantage for legumes to impose terminal differentiation on bacteroids in legumes like soybean, that lack persistent infection threads or other means of protecting some of the rhizobia from NCRs. Indeterminate nodules such as those in *Medicago* spp. have many persistent infection threads from which rhizobia are released, and these released rhizobia then enlarge mostly without undergoing cell division. Rhizobia within infection threads in *M. truncatula* do not differentiate, implying that these rhizobia are not exposed to NCRs (Mergaert et al., 2006). Therefore, the bacteria cultured from such nodules are probably those from within the infection threads, which are seen in sections of almost all infected cells.

In contrast, rhizobia released into cells of determinate nodules as in soybean do divide (Goodchild and Bergersen, 1966) and so infection threads can be much reduced and shorter compared with the complex architecture of infection threads seen e.g., in *M. truncatula* (Gage, 2004; Monahan-Giovanelli et al., 2006). Therefore, if a legume with determinate nodules acquired NCRs that induce terminal bacteroid differentiation, relatively few bacteria would be protected within infection threads. A successful symbiosis requires mutual benefit to both partners; if the legume were to impose constraints on bacteroid survival, such that there were insufficient rhizobia that could escape from senescing nodules, then a key aspect of the

symbiosis, namely rhizobial benefit, would be lost. Thus, any advantage of controlling bacterial growth by inducing terminal differentiation could be outweighed by the disadvantage of having too few rhizobia surviving the symbiosis. It remains to be established whether, in diverse legumes, there is a correlation between protection of rhizobia within infection threads and acquisition of terminal differentiation of bacteroids induced by NCR peptides.

Future Perspectives

Key questions remain with regard to understanding the functions of NCR peptides acting alone and/or in combination. One problem is that they can bind many proteins (including other NCRs), but several of the observed interactions may be spurious and have no effect in nodules. Another issue is that the targets of NCRs may be expressed only in nitrogen-fixing bacteroids, making it difficult to use rhizobial genetics to identify their targets. So how can the analysis of the physiological functions of NCRs be addressed? One approach could be to analyze NCR function in IRLC legumes that express few NCRs, but getting the molecular genetics systems established for such legumes could be difficult. Another approach as suggested (Van de Velde et al., 2010) could be to introduce NCRs from IRLC legumes into a transformable non-IRLC legume such as *L. japonicus* or a promiscuous legume such as *Phaseolus vulgaris*. Although such a transgenic legume may be compromised for rhizobial survival in nodules, this would not be a problem with laboratory-maintained plants. Such an approach could be feasible using NCR genes from an IRLC legume that has relatively few NCRs that can induce terminal bacteroid differentiation. The technology is now in place to allow legume transformation using single constructs carrying multiple genes, so it should be possible to introduce multiple NCR genes into a transformable IRLC legume such as *L. japonicus* and determine effects on bacteroid differentiation and symbiotic nitrogen fixation.

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JAD and EK discussed the ideas and edited the manuscript prior to submission. JAD drafted the manuscript. Both authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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