Inoculation- and Nitrate-Induced CLE Peptides of Soybean Control NARK-Dependent Nodule Formation

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Systemic autoregulation of nodulation in legumes involves a root-derived signal (Q) that is perceived by a CLAVATA1-like leucine-rich repeat receptor kinase (e.g. GmNARK). Perception of Q triggers the production of a shoot-derived inhibitor that prevents further nodule development. We have identified three candidate CLE peptide-encoding genes (*GmRIC1*, *GmRIC2*, and *GmNIC1*) in soybean (*Glycine max*) that respond to *Bradyrhizobium japonicum* inoculation or nitrate treatment. Ectopic overexpression of all three CLE peptide genes in transgenic roots inhibited nodulation in a GmNARK-dependent manner. The peptides share a high degree of amino acid similarity in a 12-amino-acid C-terminal domain, deemed to represent the functional ligand of GmNARK. *GmRIC1* was expressed early (12 h) in response to *Bradyrhizobium*-sp.-produced nodulation factor while *GmRIC2* was induced later (48 to 72 h) but was more persistent during later nodule development. Neither *GmRIC1* nor *GmRIC2* were induced by nitrate. In contrast, *GmNIC1* was strongly induced by nitrate (2 mM) treatment but not by *Bradyrhizobium* sp. inoculation and, unlike the other two GmCLE peptides, functioned locally to inhibit nodulation. Grafting demonstrated a requirement for root GmNARK activity for nitrate regulation of nodulation whereas *Bradyrhizobium* sp.-induced regulation was contingent on GmNARK function in the shoot.

Most legume species form a symbiotic relationship with nitrogen-fixing soil bacteria collectively called rhizobia. This occurs via a complex signaling exchange between the plant and bacteria and results in the formation of specialized root organs known as nodules (Ferguson et al. 2010). Within the nodule, the plant receives fixed atmospheric nitrogen from the bacteria in exchange for photoassimilates (Udvardi et al. 1988). Symbiotic nitrogen fixation is critical to legume cultivation because it provides both economic and environmental advantages over other crops. Additionally, nodule formation provides an excellent system to study lateral organ development, short- and long-distance signaling, and developmental regulation within plants.

Nodulation commences following the plant's perception of specialized lipooligosaccharide signal molecules produced by the bacteria called nodulation (Nod) factors (NF) (Ferguson and Mathesius 2003; Kouchi et al. 2010). Soybean (*Glycine*

max) is the most widely cultivated legume (Ferguson and Gresshoff 2009) and forms determinate type nodules in a symbiosis with *Bradyrhizobium japonicum* and *Rhizobium fredii*. In soybean, NF are perceived by LysM-type receptor kinase receptors, namely GmNFR5 (Indrasumunar et al. 2010a) and GmNFR1 (Indrasumunar et al. 2010b). Downstream signaling events in the root lead to the reinitiation of cortical cell divisions, pericycle cell divisions, and vascularization and the initiation of a lateral nodule meristem.

Early signaling events have been well-studied in model legume species, including *Lotus japonicus*, *Medicago truncatula*, *Pisum sativum*, and *G. max*. This has allowed for the identification of key components required for nodule development (Ferguson et al. 2010; Oldroyd and Downie 2008). Initial signaling events lead to calcium spiking in and surrounding the nucleus and trigger the activation of CCamK (Kosuta et al. 2008; Oldroyd and Downie 2006) which, together with the activation of a cytokinin receptor (Gonzalez-Rizzo et al. 2006; Murray et al. 2007; Tirichine et al. 2007), results in the modulation of the expression of several early nodulation genes (Heckmann et al. 2006; Schauser et al. 1999; Vernie et al. 2008).

The plant regulates the number of nodules it forms to optimize its response to both internal and external stimuli, including preexisting infection events and environmentally available nitrogen. The inbuilt mechanism is called autoregulation of nodulation (AON) (Caetano-Anollés and Gresshoff 1991a and b). This mechanism is responsible for legumes exhibiting a distinct crown nodulation phenotype. The zone affected is the region of the root that has emerging root hairs that are susceptible to rhizobia infection and cell division initiation (Bhuvaneshwari et al. 1980, 1981; Calvert et al. 1984). In soybean, this zone is sliding because AON continually permits infection and early cell division but blocks the advance toward nodule meristem formation (Calvert et al. 1984; Mathews et al. 1989). Mutants in AON remove this secondary blockage, allowing nodule formation over a larger root portion, which leads to more nodules being formed and an increased “nodulation interval” spanning the root system (Carroll et al. 1985a and b). It is of interest that, in both *P. sativum* and *M. sativa*, nodulation is blocked at an earlier stage, because the arrested nodule foci observed in soybean are not observed in these species (Caetano-Anollés and Gresshoff 1991a and b; Sagan and Gresshoff 1996).

The current model for AON (Caetano-Anollés and Gresshoff 1991a and b; Ferguson et al. 2010; Gresshoff and Delves 1986; Magori and Kawaguchi 2009) begins with the production of a root-derived cue (Q) that is produced following both the first nodulation-induced cell divisions in the root (Caetano-Anollés and Gresshoff 1990; Li et al. 2009) and further nodule maturation and functioning associated with the onset of nitrogen fixa-

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tion (Li et al. 2009) (Fig. 1). Q is subsequently transported to the shoot, presumably via the xylem stream, where it is perceived by a phloem parenchyma-localized leucine-rich repeat (LRR) receptor kinase (Nontachaiyapoom et al. 2007). This LRR receptor kinase is similar in gene and protein structure to CLAVATA1 of *Arabidopsis*, and is encoded by *GmNARK* in soybean (Searle et al. 2003) and its orthologs in other legumes: *LjHAR1* (Krusell et al. 2002; Nishimura et al. 2002a), *PsSYM29* (Krusell et al. 2002), *GsNARK* (*G. soja*) (Searle et al. 2003) and *MtSUNN* (Schnabel et al. 2005). Loss-of-function mutations in these orthologs lead to increased nodulation (termed “hyper-” and “supernodulation”), increased portion of the root showing nodule development (the nodulation interval), altered lateral root growth, and decreased sensitivity to nitrate inhibition of nodulation (Fig. 1). To date, *GmNARK* is the only genetic component that has been identified in the AON pathway of soybean (Searle et al. 2003), although related genes and nodule regulation mutants have been defined in other legumes showing altered AON: *Ljklavier* (Oka-Kira et al. 2005) *Pssym28* (Krusell et al. 2011; Sagan and Duc 1996), *Mtrdn1* (Frugoli 2010), *Psnod3* (Postma et al. 1988) *Ljtml* (Magori et al. 2009), *Ljrdh1* (Ishikawa et al. 2008), and *LjASTRAY* (Nishimura et al. 2002b). Using grafting and split-root approaches, AON and *GmNARK*/*LjHAR1*/*MtSUNN* were shown to act systemically in the shoot to regulate nodule numbers in the root (Delves et al. 1986, 1987; Jiang and Gresshoff 2002; Krusell et al. 2002; Nishimura et al. 2002a; Olsson et al. 1989; Penmetsa et al. 2003).

Following the perception of Q by NARK, a novel signal is produced that acts as a shoot-derived inhibitor (SDI) of nodu-

lation. SDI is transported from the shoot to the roots, where it prevents further nodulation events (Mathews et al. 1989). Thus far, work to identify SDI has indicated that it is likely a small molecule (less than 1,000 Da) that is not a protein, peptide, or RNA (Lin et al. 2010).

In soybean, all AON mutants isolated have been found to have mutations in *GmNARK* (Nishimura et al. 2002a; Searle et al. 2003; J. Batley, *unpublished data*). Interestingly, all AON mutants are also nitrate tolerant, because they form nodules in the presence of nitrate levels that are otherwise inhibitory to nodulation in wild-type (WT) plants (Carroll et al. 1985a and b; Penmetsa et al. 2003; Schnabel et al. 2005; Wopereis et al. 2000). That *GmNARK* mutations affect both AON and nitrate regulation of nodulation indicates that this gene is a common component of both regulatory mechanisms. This would also appear to explain why *GmNARK* expression is observed in the phloem parenchyma of both the shoot and the root (Nontachaiyapoom et al. 2007) but, in contrast to *AtCLAVATA1*, not in the root or shoot apical meristem. Furthermore, recent work has shown that both local and systemic regulation of nodulation occurs in response to nitrate (Jeudy et al. 2010).

The structural similarity of *GmNARK* to *CLAVATA1* in *Arabidopsis* led to the proposal (Searle et al. 2003) that the ligand of *GmNARK* may be a CLE peptide, similar to the *CLV3* ligand that binds to *CLAVATA1* (Clark et al. 1997; Fletcher et al. 1999; Kondo et al. 2006, 2008; Ogawa et al. 2008). CLE peptides are required for several plant developmental and regulatory processes, including for the regulation of the shoot (Fletcher et al. 1999) and root (Fiers et al. 2004) apical meristems and for vasculature differentiation (Hirakawa

Fig. 1. Proposed model of NARK-dependent CLE activity in the root and shoot. Autoregulation of nodulation involves long-distance signaling requiring the interaction of RIC1 or RIC2 with NARK in the leaf and the subsequent inhibition of nodulation via the production of a shoot-derived inhibitor (SDI). Local nitrate inhibition of nodulation is established by the interaction of NIC1 with NARK in the root leading to production of an SDI-like nitrate induced inhibitor (NII) of nodulation.

et al. 2010; Ito et al. 2006). CLE peptides are characterized by an N-terminal signal peptide and a C-terminal region of approximately 12 to 13 amino acids that is proposed to act as the final active product (Oelkers et al. 2008).

Significant modification of CLE peptides occurs post-translationally, including proteolytic cleavage (Ni and Clark 2006; Ni et al. 2010), hydroxylation at key prolines (Kondo et al. 2006; Ohyama et al. 2009), and glycosylation (arabinose addition at proline 7) (Ohyama et al. 2009; Shinohara and Matsubayashi 2010). There is some debate in the literature but the best evidence indicates that CLV3 functions as a 12- or 13-amino-acid triple- β -1,2-linked arabinosylated peptide (Kondo et al. 2006; Ohyama et al. 2009).

To date, CLE peptides have been identified that are induced specifically by rhizobia or by either rhizobia or nitrate. All reduce nodulation in *L. japonicus* and *M. truncatula* when ectopically overexpressed in transgenic roots. Whereas the *L. japonicus* peptides only reduced nodulation effectively in WT plants (Okamoto et al. 2009), the *M. truncatula* peptides also exhibited partial inhibition in hypernodulating *Mtsunn-1* plants (Mortier et al. 2010). However, a complete lack of nodule suppression which was not seen in the *Mtsunn-1* background was observed when *MtCLE12* was overexpressed using another allele of *Mtsunn* (*Mtsunn-4*) (I. Saur, N. Imin, and M. Djordjevic, personal communication). Furthermore, the large number of LRR receptor-like proteins (Diévar and Clark 2004) and CLE peptide ligands (Oelkers et al. 2008) suggests that additional CLE peptides and LRR receptor partners may regulate plant development.

We investigated the function of CLE peptides in the regulation of soybean nodulation, and identified three CLE peptide genes that exhibited either *Bradyrhizobium* Nod factor- or nitrate-induced gene expression. We designated these genes as rhizobia-induced CLE (*RIC1* and *RIC2*) and nitrate-induced CLE (*NIC1*). We show that overexpression of the inoculation-dependent CLE genes (*RIC1* and *RIC2*) inhibited soybean nodulation in both a systemic and NARK-dependent manner. In contrast, overexpression of the nitrate-responsive CLE (*NIC1*) only partially reduced nodulation, and appeared to function in a local yet still NARK-dependent manner. Grafting experiments using WT and *nark* mutant plants grown under several nitrate conditions confirmed this local role for NARK in nitrate regulation of nodulation. We also showed differences in the localization of the nodule inhibition responses induced by inoculation- or nitrate-responsive CLE peptides. We propose that these differences may account for the different yet overlapping roles of nitrate- and inoculation-dependent regulation of nodulation in soybean.

RESULTS

Identification of *Bradyrhizobium* inoculation- and nitrate-responsive CLE peptides.

To identify candidates for inoculation- or nitrate-dependent CLE peptides involved in nodule regulation, searches were undertaken based on sequence homology with the *LjCLE-RS1/2* protein sequences (Okamoto et al. 2009). BLAST searches identified two *M. truncatula* genes upregulated in response to inoculation in the *Medicago* gene atlas (Benedito et al. 2008), which have subsequently been shown to reduce nodulation when overexpressed (Mortier et al. 2010). The peptide sequence of all rhizobia inoculation-dependent legume CLE peptides was aligned using ClustalX2 to produce a consensus sequence (Larkin et al. 2007). Publicly available soybean genome sequences (Phytozome) (Schmutz et al. 2010) and expressed-sequence tag sequences (National Center for Biotechnology Information [NCBI]) were searched to identify soybean se-

quences sharing homology with this consensus sequence or with the *L. japonicus* and *M. truncatula* CLE sequences. Searches focused on genes that shared the greatest similarity with the C-terminal CLE domain. Three soybean CLE genes were subsequently identified as candidates for either *Bradyrhizobium* sp. inoculation or nitrate response. These genes were designated *RIC1*, *RIC2*, and *NIC1* (Figs. 2 and 3).

Further BLAST searches conducted in soybean identified one highly similar homolog for each of the three CLE genes likely resulting from the ancestral soybean allopolyploidization event (Fig. 2). Primers specific to each of the genes have indicated that the duplicate copies in each case are not tran-

Fig. 2. Microsynteny and homology between *GmCLE* genes. Amino acid conservation (percent identity and percent similarity) between each of the CLE genes was highest between each gene (red) and its inactive duplicate (gray) and between the inoculation-responsive CLEs **A**. Phytozome cluster analysis showed a well conserved region of synteny exists between species for **B**, *RIC1* or *RIC2* and **C**, *NIC1*. Aly, *Arabidopsis lyrata*; Gma, *Glycine max*; Cpa, *Carica papaya*; Ath, *A. thaliana*; Ptr, *Populus trichocarpa*.

scriptionally active in the tissue and treatment types tested in this study based on reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analyses (data not shown). *RIC1* and *NIC1* are located in syntenous regions of chromosome 13 and 12, respectively. In each case, the duplicate is located on the syntenous chromosome region adjacent to the actively expressed CLE (Fig. 2). *RIC2* has a homolog in a syntenous region of chromosome 12 independent of this locus (Fig. 2). The degree of protein similarity or identity suggests that *RIC1*, *RIC2*, and *NIC1* belong to an ancient CLE peptide gene family that diverged (by 50 to 80%) but then duplicated as part of one of the genome-wide polyploidization events that occurred 59 and 13 million years ago (Schmutz et al. 2010). Subsequent divergence (12 to 15%) and genome dispersion has since led to their current sequence state. Remarkably, the 12-residue C-terminal region remained conserved, suggesting functional constraints.

Sequence characteristics of the *Bradyrhizobium* inoculation-induced CLE peptides.

Peptide elicitors of AON are predicted to require export from the producer cell to the xylem that is dependent on an N-terminal signal peptide. SignalP 3.0 analysis (Bendtsen et al. 2004) predicted that *RIC1*, *RIC2*, and *NIC1* possess an N-terminal signal peptide which comprises approximately 30 hydrophobic amino acids, with the most likely cleavage site for the signal peptide being after 28, 29, and 26 amino acids for *RIC1*, *RIC2*, and *NIC1*, respectively (Fig. 3A). This analysis also showed that the signal peptide region of the inoculation-dependent CLE peptides shared areas of high conservation, including a well-conserved motif identified by Okamoto and associates (2009) (at least four of the seven legume CLE sequences were conserved at each residue) of 'FSTFFMTLQARSL' encompassing the predicted signal peptide cleavage site. *NIC1* showed

Fig. 3. Multiple sequence alignment of legume inoculation and nitrate responsive CLEs **A**, Predicted signal peptide of the soybean CLE peptides is underlined in black, the conserved signal peptide residues altered in *NIC1* are enclosed by a red box, and the conserved 12-amino-acid CLE domain is enclosed by a pink box. **B**, A logo alignment (Crooks et al. 2004) of the CLE domain and C-terminal extension illustrates the degree of conservation at each residue.

the most significant divergence from this motif because it lacks several of the nucleophilic and hydrophobic amino acids. Two well-conserved proline residues, outside of the canonical 12-amino-acid CLE peptide region (Oelkers et al. 2008), were also identified close to the C-terminus and are conserved between *L. japonicus* and *M. truncatula* (Fig. 3A and B); their function is currently obscure.

Expression pattern of *GmCLE* genes.

Soybean primary root tissue corresponding to the area of root-hair emergence at the time of *Bradyrhizobium* sp. inoculation was harvested in a time-course manner to identify genes responding to early infection events (S. Hayashi, B. Ferguson, and P. Gresshoff, unpublished data). RT-qPCR studies using this tissue showed that *RIC1* expression was significantly upregulated in response to inoculation with effective WT *Bradyrhizobium* spp. relative to the ineffective *nodC*⁻ mutant (Fig. 4A), which is unable to produce Nod factor because of a chitin synthase deficiency (Debellé et al. 1992; Sanjuan et al. 1992). This difference in expression was significant as early as 12 h after inoculation and increased until the last tissue collection time in this study at 72 h. It also demonstrates NF dependence for *RIC1* expression. In contrast, more mature, nodulated root tissue (14 days after inoculation) showed low *RIC1* expression (Fig. 5). *NIC1* expression also responded slightly to inoculation; however, this was not detectable until 48 h after inoculation and was markedly weaker relative to *RIC1* expression levels (Fig. 4A). *RIC2* expression was not significantly changed until 72 h after inoculation (Fig. 4a) but was induced thereafter because mature, nodulated root tissue (14 days after inoculation) showed high *RIC2* expression (Fig. 5).

In an initial study to determine whether nitrate can induce the expression of the soybean CLE genes, root tissue was harvested after being treated for 2 weeks with either 0 or 10 mM KNO₃. Using RT-qPCR, *NIC1* expression was significantly induced by nitrate relative to the control, whereas *RIC1* and *RIC2* expression remained unaffected (Supplementary Fig. S1). A temporal analysis to identify the induction of *NIC1* expression by nitrate was subsequently performed using root tissue harvested at 0, 8, or 24 h after the commencement of 10 mM KNO₃ treatments. After 8 h, *NIC1* expression was significantly elevated and increased further by 24 h (Fig. 4B).

To determine whether *NIC1* expression correlated with the inhibition of nodulation caused by nitrate, various nitrate concentrations were tested. Soybean plants were treated with either 0, 2, 5, 10, or 15 mM KNO₃ for 2 days prior to and 2 weeks following *B. japonicum* CB1809 inoculation. The treated plants were then harvested concurrently for RT-qPCR and nodule count analyses. *NIC1* was significantly induced by KNO₃ at levels as low as 2 mM and reached a plateau after the 5 mM treatment, while nodule numbers per plant were reduced in an inverse manner (Fig. 5). In contrast to the early inoculation time course data, *RIC1* expression did not appear to correlate with mature nodule numbers (and was significantly lower in this mature tissue), whereas *RIC2* expression correlated strongly, exhibiting reduced expression levels at higher nitrate concentrations (Fig. 5).

Similar to nodulation, the systemic regulation of root arbuscular mycorrhizal infection acts through GmNARK in the shoot (Meixner et al. 2005, 2007). To determine whether any of the three soybean CLE genes were upregulated by arbuscular mycorrhizal infection and, thus, had a role in their regulation, RT-qPCR was performed using cDNA from *Glomus intraradices*-infected soybean roots (cDNA provided by S. Schaarschmidt and B. Hause, Halle, Germany). No significant change in the expression of the CLE genes relative to that detected in uninfected plants was observed at 5, 14, or 70 days after infection

with *G. intraradices* in either WT Bragg or *nts1116* hyper-nodulating mutant plants.

Cloning and transgenic expression of *GmCLE* genes.

To determine whether the soybean CLE peptides regulate nodulation in a NARK-dependent manner, a reverse-genetics approach employing ectopic overexpression in a chimeric hairy-root system was used. Overexpression of the systemic Q signal would be expected to inhibit nodulation, even in non-transformed roots. To test this, the complete coding sequences (CDS) of *RIC1*, *RIC2*, or *NIC1* were cloned into the pKANNI-BAL vector for the expression of efficient RNAi constructs (Wesley et al. 2001). However, to generate overexpressing

Fig. 4. Gene expression of soybean CLE genes in the zone of nodulation after rhizobia inoculation **A**, Plants were inoculated with compatible (wild-type [WT]) or incompatible (*nodC*⁻) rhizobia before gene expression was quantified using reverse-transcription quantitative polymerase chain reaction. Error bars indicate mean $\pm$ standard error (SE) of two biological replicates ($n = 6$). *RIC1* was increased as early as 12 h after inoculation with WT bacteria whereas *RIC2* was not induced until later time points. *NIC1* was only slightly induced, though not significantly, and was not detectable until later. Gene expression of *NIC1* in the root after nitrate treatment was significantly increased as early as 8 h after treatment with 10 mM KNO₃ and increased further at 24 h **B**, Error bars indicate mean $\pm$ SE ($n = 4$).

constructs, the antisense construct portion was omitted and the intron left intact, because previous studies have shown that the inclusion of an intron can lead to higher and more consistent expression levels in transgenic plants (Norris et al. 1993).

Triparental mating was used to integrate the overexpression constructs into *Agrobacterium rhizogenes* K599, which efficiently induces the formation of transgenic hairy roots in soybean (Kereszt et al. 2007). An integrative vector was used because they have been shown to increase the transformation efficiency relative to that of binary vectors, which are commonly used in plant transformation (Hayashi et al. 2008). RT-qPCR analysis indicated that significant overexpression was achieved with each of these constructs (Supplementary Table S2). Overexpression of either *RIC1* or *RIC2* inhibited nodulation (*RIC1*: 0 nodules, *RIC2*: 0.6 nodules, and control: 9.45 nodules per root) in WT soybean plants. In contrast, there was no reduction in the number of nodules on equivalent *nark* (*nod4*, V370D) plants relative to vector-only transformed control *nark* plants (Fig. 6A). This indicates that nodule inhibition by CLE peptide overexpression is GmNARK dependent. Similarly, *NIC1* overexpression significantly reduced nodulation ($P < 0.05$), although this reduction was not as strong as that observed for the *RIC* genes. As was the case for *RIC1* and *RIC2*, this reduction in nodulation was not observed in *nod4* plants (Fig. 6B).

To determine whether this nodule inhibition was a systemic effect, the experiment was repeated with chimeric plants where both hairy roots and the primary root system were maintained. Nodulation was assessed on both transgenic roots and the WT primary root system. The two *RIC* genes inhibited nodulation on both the hairy roots and the primary roots in a systemic manner (Fig. 7). In contrast, *NIC1* inhibited nodulation on the hairy roots while the primary roots were unaltered relative to nodulation on the vector-transformed control plants (Fig. 7).

Nitrate regulation of nodulation.

The strong systemic effect of AON has complicated the understanding of nitrate control of nodulation. Several studies have indicated that nitrate regulation is determined by the shoot (Day et al. 1989; Francisco and Akao, 1993), whereas others have highlighted the role of a local regulation in the root (Carroll and Gresshoff 1983; Carroll and Mathews 1990; Hinson 1975). In fact, both acting in parallel have recently been shown to be important for nodulation (Jeudy et al. 2010) and may regulate nodulation differently.

To determine what role NARK plays locally in nitrate regulation of nodulation, we investigated the nodule numbers of WT (Williams82) and supernodulation (*nod4*) (*nts*) plants that were grafted and treated with high or low levels of nitrate. Plants with an intact AON response (WT/*nts* and WT/WT) were used to assess whether NARK functions in the root as part of a local nitrate-controlled regulation of nodulation.

When treated with 5 or 10 mM KNO_3 , nodule numbers were reduced significantly ($P = 0.02$ and 0.002 , respectively) in grafted plants having a WT rootstock compared with those having a *nod4* mutant rootstock (Fig. 8). There was no significant difference in nodule numbers when no nitrate was applied ($P = 0.23$). In concordance with previous studies (Delves et al. 1986, 1987), there was no difference observed in nodule numbers between the AON-defective grafting combinations (*nts*/WT and *nts*/*nts*) at any nitrate concentration (data not shown).

Defining a putative CLE peptide-binding site in the GmNARK LRR domain.

Using the Phyre protein structure prediction server (Kelley and Sternberg 2009), a model of the GmNARK LRR domain was determined for visualization using PyMOL (DeLano 2002). This model suggested a boomerang-shaped structure (Fig. 9A).

Fig. 5. CLE expression relative to nitrate concentration and nodule numbers. Tissue samples were harvested 2 weeks following inoculation and 3 weeks following nitrate treatment. Error bars indicate mean $\pm$ standard error ($n = 3$). *NIC1* expression was significantly induced by 2 mM nitrate and plateaued at 5 mM. *RIC2* correlated strongly with nodule numbers, which were significantly reduced as nitrate concentration increased. *RIC1* did not appear to correlate with nodulation at this later stage of development.

Two missense mutants showing strong supernodulation phenotypes reminiscent of nonsense mutants in the *GmNARK* gene were positioned adjacent to a pronounced cleft in the central region of the LRR region (Fig. 9A). Despite being 24 amino acids apart (L346F and V370D) (J. Batley, *unpublished data*), both amino acids were adjacent in the predicted structure and directly flank the cleft. Though the detailed structure of the GmCLE peptides remains unknown, they may fit within the modeled cleft region. Absence of suppression of nodulation in *nod4* plants transformed to overexpress the *CLE* genes further suggests that this region defines the putative CLE peptide-binding site.

DISCUSSION

Based on homology to CLE peptides that are necessary for nodule regulation in other legumes, we identified three inoculation- or nitrate-responsive CLE peptides in soybean. These three CLE peptides regulate soybean nodulation in a NARK- and NF- or nitrate-dependent manner, although there are differences in their expression response and in the localization of their action (Table 1). The soybean CLE peptides share

a high level of conservation in the CLE domain and have well-conserved motifs within the signal peptide and at the C-terminal.

Gene expression analyses showed that *RIC1* and *RIC2* are induced in response to Nod factor produced by compatible *B. japonicum* inoculation; however, differences in the timing of their induced expression suggest that they are not wholly redundant. *RIC1* is expressed early, possibly in response to the initial signaling and cell division events induced following *Bradyrhizobium* sp. inoculation. This expression pattern is consistent with that of *LjCLE-RS1/2* and *MtCLE13*, which are reported to respond quickly to inoculation (Mortier et al. 2010; Okamoto et al. 2009). In the case of *MtCLE13*, expression was also observed in the *ERN1* mutant *bit1-1* (Mortier et al. 2010), which is defective in the later stages of Nod factor signaling (Andrianakaja et al. 2007; Middleton et al. 2007). In contrast, *RIC2* expression correlates with the emergence of more mature nodule structures, similar to the expression of *MtCLE12* (Mortier et al. 2010). Therefore, it may be induced following the onset of the nodule meristem or in response to nitrogen fixation. That multiple signals may act in parallel to regulate nodule numbers is not surprising in light of the findings by Li

Fig. 6. Overexpression of inoculation induced CLE genes **A**, Hairy roots induced with the vector-only control showed a normal nodulation phenotype on Williams wild-type (WT) plants whereas nodules were significantly inhibited ($P < 0.05$) in WT roots overexpressing *RIC1* or *RIC2*. In contrast, nodule numbers were not reduced in *nod4* plants. Error bars indicate mean $\pm$ standard error (SE) ($n = 15$). **B**, Overexpression of nitrate-induced CLE gene. Hairy roots induced with the vector-only control showed a normal nodulation phenotype on Williams WT plants whereas nodules were significantly reduced ($P < 0.05$) in WT roots overexpressing *NIC1*. Nodule numbers were not reduced in the *nod4* plants overexpressing *NIC1* relative to controls. Error bars indicate mean $\pm$ SE ($n = 8$).

and associates (2009) that AON in pea may be activated at several developmental stages of nodulation.

Overexpression of both *RIC1* and *RIC2* was sufficient to elicit a systemic AON response and, in both cases, greatly reduced nodulation in WT plants. The complete lack of nodule

inhibition in *GmNARK* mutant plants suggests that these CLE peptides are functioning in AON through NARK, possibly as the Q signal described in various AON models (Ferguson et al. 2010; Magori and Kawaguchi, 2009). Future experiments aimed at directly detecting the transport and function of these

Fig. 7. Nodule numbers of plants having both untransformed primary roots and hairy roots overexpressing inoculation- or nitrate-induced CLE genes. Vector-transformed control plants showed no difference in nodulation between the hairy roots and the primary roots. Overexpressing *RIC1* or *RIC2* reduced nodulation on both the hairy roots and the primary roots, indicating a systemic inhibitory effect. In contrast, while nodule numbers were significantly reduced ($P < 0.05$) on hairy roots overexpressing *NIC1*, no reduction was observed on the primary roots, indicating that *NIC1* functions locally. Error bars indicate mean $\pm$ standard error ($n = 6$).

Fig. 8. Grafting of Williams (wild-type [WT]) scions with *nod4* rootstocks under different nitrate conditions. Grafted plants having WT rootstock had significantly fewer nodules when treated with either 5 or 10 mM nitrate compared with those having *nod4* rootstocks or those not treated with nitrate. Error bars indicate mean $\pm$ standard error ($n = 5$).

CLE peptides will help to verify these models. That the legume *CLE* genes have different expression patterns and timing appears to indicate that NARK and its orthologs in other legumes have evolved as a common receptor in various nodule-regulation mechanisms. This would explain the dual role of NARK in AON and nitrate regulation of nodulation (Carroll et al. 1985a and b; Ferguson et al. 2010; Kouchi et al. 2010). That none of the CLE genes were regulated by AMF infection, despite this symbiosis also being regulated through NARK (Meixner et al. 2005, 2007), may indicate that other CLE peptides are involved in mycorrhizal control.

It appears that subtle differences exist between the CLE peptides identified in each of the three legume species in the manner in which inoculation- and nitrate-induced CLE peptides function. *LjCLE-RS2* is reported to be responsive to both

nitrate treatment and rhizobia inoculation and to exhibit a systemic regulation response when overexpressed in *L. japonicus* hairy roots (Okamoto et al. 2009). This led to the proposal that *LjCLE-RS2* could systemically induce nodule regulation in response to both inoculation and nitrate in *L. japonicus*. In contrast, *NIC1* was induced in response to nitrate but was not substantially altered by *Bradyrhizobium* spp. Moreover, it appears to function locally in the root and not systemically. Significant *NIC1* expression was detected at the lowest nitrate levels we tested (2 mM KNO₃) and reached a plateau at 5 mM KNO₃. Although there was a small increase in *NIC1* expression 48 h after *Bradyrhizobium* sp. inoculation, the increase was minimal compared with that detected following nitrate treatment. It was also dramatically lower than that detected for *RIC1* following *Bradyrhizobium* sp. inoculation. The *NIC1* ex-

Fig. 9. Phyre modeling (Kelley and Strenberg 2009) (based on PDB: 1ZIW) of the NARK leucine-rich repeat domain revealed a boomerang-like structure with a distinct central cleft. **A**, Site of two amino acid residues (L346 and V370) that, when mutated, cause a supernodulation phenotype are shown flanking the central cleft and define the likely ligand binding site. **B and C**, Resulting supernodulation phenotypes caused by the L346 and V370 mutations of the *nod3-7* and *nod4* mutants, respectively.

Table 1. Comparison of legume CLEs capable of nodule regulation^a

Characteristic	<i>Glycine max</i>			<i>Medicago truncatula</i>		<i>Lotus japonicus</i>	
	RIC1	NIC1	RIC2	CLE12	CLE13	CLE RS1	CLE RS2
Nitrate induced	No	Yes	No	NA	NA	Inhibited	Yes
Early inoculation	Yes	No	No	No	Yes	Yes	Yes
Late inoculation	No	No	Yes	Yes	Yes	NA	NA
NARK/SUNN/HAR1 dependent inhibition	Yes	Yes	Yes	Allele specific	Allele specific	Yes	Yes
Systemic activity	Yes	No	Yes	Yes	Yes	Yes	Yes
CLE domain 3' extension	Yes	No	Yes	No	Yes	Yes	Yes
Signal peptide consensus sequence	Yes	Partial	Yes	Yes	Yes	Yes	Yes

^a NA = not analyzed.

pression pattern following inoculation may result from the evolutionary divergence of the CLE sequences and some conservation in their regulatory sequences. However, the reduced expression levels observed relative to *RIC1* indicate it is not likely to be involved in inoculation-dependent nodule regulation. Overexpression of *NIC1* caused an approximate 50% reduction in nodulation, indicating that it is capable of nodule inhibition, although not to the same degree as the inoculation-induced CLE genes. Consistent with the *Bradyrhizobium* sp.-induced CLE peptides, *NIC1* inhibition of nodulation was NARK dependent.

We reexamined the data from Delves and associates (1986) that investigated the grafting of AON-deficient plants in nitrate conditions and found that there may have been a nitrate-induced effect of the root-genotype on nodulation that was masked by the lack of AON in the shoot. The grafting experiments that we carried out confirmed this and showed that NARK acting in the root may regulate nodulation locally in addition to the systemic regulation of nodulation. Taken together with the *NIC1* overexpression results, these grafting experiments highlight the local role for NARK in nitrate regulation of nodulation and indicate that *NIC1* is likely a local inducer of NARK-dependent nodule regulation independent of the systemic AON mechanism. The reduced nodule inhibition response of *NIC1* may be due to this local role in nodule inhibition and a reflection of successful transformation events or to differences in the signal peptide or CLE domain that may reduce the effectiveness of processing or activity of *NIC1*.

Increasingly, it appears that motifs outside of the CLE domain are required for the in planta function and specificity of CLE peptides. CLE activity is likely related to tissue specificity of the promoters, tissue localization caused by signal peptide trafficking, and post-translational modifications of the final peptide. The receptor specificity is likely due to the distinct structure of each CLE peptide (Meng et al. 2010), where most CLE peptides appear to share at least basic structural similarities due to a bend imparted by central glycine and proline residues (Cock and McCormick 2001). The putative Q CLE peptides have distinct double central glycine residues flanked by proline, which may impart AON receptor specificity. In *Arabidopsis*, CLE function is dependent on motifs outside of the CLE domain, particularly in the signal peptide (Meng et al. 2010). This is consistent with findings regarding the activity of CLE peptides produced by soybean cyst nematodes (Wang et al. 2010). Our work in soybean indicates that the signal peptide may be critical in differentiating the tissue specificity of the nitrate-responsive CLE from the inoculation-responsive CLE peptides. Aligning the sequences of the *Bradyrhizobium* sp.-induced CLE peptides to the previously reported inoculation-dependent CLE sequences allowed us to extend the conserved motif (FSTFFMTLQARSL) within the signal peptide that was first identified by Okamoto and associates (2009) and has not previously been reported outside of legumes. We found that this motif was common among the legume CLE peptides studied whereas, in *GmNIC1*, it was only partly conserved. Interestingly, the inoculation-dependent soybean CLE peptides and *MtCLE13* also possessed the conserved proline residues close to the C-termini observed in *LjCLE-RS1/2* (Okamoto et al. 2009) though the potential function of these residues remains unclear.

Previous studies have shown that nitrate treatment of one side of a split root system does not systemically change the nodule mass on the other side of the split root system (Tanaka et al. 1985). However, in the same experiment, nitrogen fixation was systemically reduced. This regulation may be due to asparagine transport in the phloem (Suliman et al. 2010) and may explain the partial sensitivity of *nts* rootstock to nitrate in

these experiments. We have extended this understanding of a local role of nitrate regulation in legume nodulation by demonstrating a local requirement for NARK in nodulation regulation that is dependent on *NIC1*. This appears to account for why NARK is expressed in the root (Nontachaiyapoom et al. 2007), despite it previously being believed to only be effective in the shoot. Our grafting experiments also demonstrate that nitrate regulation of nodulation acts locally through NARK in the root, because the presence of NARK in the shoot alone is not sufficient to regulate nodulation following nitrate treatment. The absence of several residues in the *NIC1* signal peptide that are conserved in the inoculation-dependent CLE peptides may be responsible for its lack of systemic response. This may vary between legume species because it has been reported that the nitrate-responsive CLE peptide, *LjCLE-RS2*, can inhibit nodulation systemically in *L. japonicus*. Future domain swap experiments will be required to clarify the role of the signal peptide and other important domains within these CLE genes.

We propose an enhanced AON model in soybean that supports the recently reported model by Ferguson and associates (2010) where there is both a systemic and local role for NARK involving multiple CLE peptide ligands (Fig. 1). Future experiments will focus on detecting the peptides in planta to identify both their active and transport forms, in addition to studies aimed at better understanding the role of NARK and *NIC1* in the nitrate-dependent regulation of nodulation.

MATERIALS AND METHODS

General plant and bacterial growth conditions.

Soybean (*Glycine max*) lines used include the WT Williams82 and its isogenic supernodulating *nark* mutant lines, *nod4* and *nod3-7*, and the WT Bragg and its isogenic hypernodulating *nark* mutant line, *nts1116*. Plants were grown in controlled glasshouse conditions (28 and 26°C, day and night, respectively, with a 16-h day) in autoclaved pots and vermiculite where sterile growing conditions were required. Seed requiring sterilization were treated with 70% ethanol and 3% H₂O₂ for 1 min, followed by several rinses with water. Plants were watered as required with a modified nutrient solution lacking nitrogen (Herridge 1982). Plants were inoculated at an optical density at 600 nm of approximately 0.01 with *B. japonicum* CB1809 or the corresponding NF mutant *nodC*⁻ grown in yeast-mannitol broth at 28°C for 2 days. For nitrate treatments, plants were watered with the indicated solutions of KNO₃ every 2 days. For grafting experiments, reciprocal grafting was carried out 8 days after sowing and the graft unions were allowed to recover for 6 days before *B. japonicum* inoculation. Nodulation was scored 3 weeks after inoculation. A Student's *t* test was used to determine the statistical significance of nodulation differences.

Bioinformatic analysis.

Candidate inoculation- or nitrate-dependent CLE genes were identified by BLASTp searches of the *Medicago* expression atlas (Benedito et al. 2008) and soybean resources at NCBI and available through the Soybean Genome Project (Schmutz et al. 2010). The conserved C-terminal CLE motif of *LjCLE-RS1/2* was used as the initial query sequence. Further searches were undertaken using the initial candidates to identify additional gene candidates and their genomic environment. Where partial sequences were obtained, the complete CDS was predicted from gene models available via phytozome or as determined by gene prediction programs (Burge and Karlin 1997; Salamov and Solovyev 2000). Multiple sequence alignments (Clustal X2) (Larkin et al. 2007) and signal peptide predictions (SignalP 3.0) (Bendtsen et al. 2004) were carried out

using the soybean peptide sequences. Cluster analysis was obtained for each of the CLE genes from phytozome (Soybean Genome Project). Molecular modeling of the NARK LRR domain was carried out using residues 1 to 601 of the NARK protein (GenBank: AAN74865.1) at the Phyre web server (Kelley and Sternberg 2009) before visualization in PyMOL (DeLano 2002). The best-ranked hit for Phyre threading was to the humal toll-like receptor 3 ectodomain (PDB id: 1ziw).

Molecular biology.

The full-length CDS of *RIC1* (Glyma13g36830), *NIC1* (Glyma12g33660), and *RIC2* (Glyma06g43680) was directionally cloned into the pKANNIBAL vector for expression of RNAi constructs (Wesley et al. 2001). *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.) was used to amplify PCR products incorporating restriction nuclease sites from cDNA samples shown to express the target gene. *XhoI/EcoRI* (forward) and *KpnI* (reverse) restriction sites were included in the primer sequences depending on internal restriction sites of each gene (full primer sequences are included in Supplementary Table S1). Likely clones were confirmed by direct DNA sequencing and capillary separation. Constructs were subcloned into p15SRK2 or pM1KCK1 integration vectors (A. Kereszt, unpublished) as a *NotI* fragment before triparental mating to introduce the constructs into *A. rhizogenes* K599. *A. rhizogenes* was subsequently used for the induction of transgenic soybean hairy roots according to Kereszt and associates (2007), with slight modification for the chimeric plants, where the primary root system was not removed.

RT-qPCR analysis.

For gene-expression analyses, tissue was snap frozen and homogenized in liquid nitrogen using a mortar and pestle. RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen, San Diego, CA, U.S.A.). DNase treatment (Fermentas, Burlington, Canada) was performed on 1 µg of RNA in the presence of MgCl₂. cDNA was synthesized with SuperScript III (Invitrogen) from 0.5 µg of DNase-treated RNA. RT-qPCR was conducted using an ABI 7900HT cyclor in 384-well plates with SYBR green fluorescence detection. All reactions were performed in duplicate for at least three biological replicates using intron-spanning primers where possible and a target amplicon of approximately 100 bp. Gene expression values were calculated relative to ATP synthase or *GmCons6* (Libault et al. 2008) after normalization for PCR efficiency and plot correlation (R^2) values of each primer pair as determined by LinRegPCR 7.5 (Ramakers et al. 2003). A Student's *t* test was used to determine the statistical significance of differences in the relative expression levels.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Phytozome *Glycine max* (soybean) database: www.phytozome.net/soybean