

RESEARCH PAPER

Structure–function analysis of the *GmRIC1* signal peptide and CLE domain required for nodulation control in soybean

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Abstract

Legumes control the nitrogen-fixing root nodule symbiosis in response to external and internal stimuli, such as nitrate, and via systemic autoregulation of nodulation (AON). Overexpression of the CLV3/ESR-related (CLE) pre-propeptide-encoding genes *GmNIC1* (nitrate-induced and acting locally) and *GmRIC1* (*Bradyrhizobium*-induced and acting systemically) suppresses soybean nodulation dependent on the activity of the nodulation autoregulation receptor kinase (*GmNARK*). This nodule inhibition response was used to assess the relative importance of key structural components within and around the CLE domain sequences of these genes. Using a site-directed mutagenesis approach, mutants were produced at each amino acid within the CLE domain (RLAPEGDPDHHN) of *GmRIC1*. This approach identified the Arg1, Ala3, Pro4, Gly6, Pro7, Asp8, His11, and Asn12 residues as critical to *GmRIC1* nodulation suppression activity (NSA). In contrast, none of the mutations in conserved residues outside of the CLE domain showed compromised NSA. Chimeric genes derived from combinations of *GmRIC1* and *GmNIC1* domains were used to determine the role of each pre-propeptide domain in NSA differences that exist between the two peptides. It was found that the transit peptide and CLE peptide regions of *GmRIC1* significantly enhanced activity of *GmNIC1*. In contrast, the comparable *GmNIC1* domains reduced the NSA of *GmRIC1*. Identification of these critical residues and domains provides a better understanding of how these hormone-like peptides function in plant development and regulation.

Key words: Autoregulation of nodulation, CLE peptides, legumes, nodulation, soybean, symbiosis.

Introduction

In agricultural systems, reduced nitrogen is often limiting and thus requires application of nitrogen fertilizer, which has both cost and environmental concerns (Jensen *et al.*, 2012). Most legume species develop a symbiotic relationship with soil rhizobia that reduces the need for this input due to biological nitrogen fixation. Rhizobia undergo differentiation to bacteroids and are housed in a complex organ, known as a nodule, which maintains the conditions required for nitrogen fixation to occur. The nodule develops on the roots through a re-initiation of cell divisions and concurrent infection events (reviewed by Ferguson *et al.*, 2010; Desbrosses and Stougaard, 2011).

The development of nodules is regulated by the plant in response to internal and external cues, including available reduced nitrogen, and through a systemic regulatory mechanism known as the autoregulation of nodulation (AON; first proposed by Gresshoff and Delves, 1986). AON is established in response to early nodulation signalling events through long-distance signals between the root and shoot (Delves *et al.*, 1986; Li *et al.*, 2009; Reid *et al.*, 2011b) and is maintained by the nodulation autoregulation receptor kinase (*GmNARK*) in soybean (Searle *et al.*, 2003). *GmNARK* is structurally similar to the CLAVATA1 (CLV1) receptor kinase of *Arabidopsis* (Clark *et al.*, 1997), and is functionally

conserved with its homologues from other legume species (Krusell *et al.*, 2002; Nishimura *et al.*, 2002; Schnabel *et al.*, 2005).

In *Arabidopsis*, the leucine-rich repeat-receptor kinase (LRR-RK) CLAVATA1 regulates the stem cell population of the shoot apical meristem (SAM) through direct perception of the small peptide CLV3 (Fletcher *et al.*, 1999; Ogawa *et al.*, 2008) in a negative feedback loop with the transcription factor WUSCHEL (WUS; Brand *et al.*, 2000; Schoof *et al.*, 2000). Both the CLV3 peptide and WUS protein may act in cells at a distance from their origin of production via intercellular movement (Lenhard and Laux, 2003; Yadav *et al.*, 2011). Many similarities exist between the functional components of the CLV system and AON (reviewed in Reid *et al.*, 2011b), including the recent identification of a *WUSCHEL-RELATED HOMEBOX* gene (*WOX5*; Osipova *et al.*, 2012), a *KINASE-ASSOCIATED PROTEIN PHOSPHATASE* gene (*KAPPI*; Miyahara *et al.*, 2008), CLAVATA2-receptor protein-controlled supernodulation (Krusell *et al.*, 2011), and CLE (CLV3/ESR-related) peptides.

CLE peptides functioning in the *Arabidopsis* CLV system regulate the stem cell population of the SAM via CLV1, whereas those of AON regulate nodulation in a *GmNARK*-dependent manner (Gresshoff *et al.*, 2009; Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Reid *et al.*, 2011a). In soybean, three such CLE peptides have been characterized (Lim *et al.*, 2011; Reid *et al.*, 2011a). They are induced in response to either nitrate (*GmNIC1*) or early infection and nodule establishment events in the root zone susceptible to nodulation (*GmRIC1* and *GmRIC2*; Hayashi *et al.*, 2012). *GmRIC1* and *GmRIC2* inhibit nodulation in a systemic manner when constitutively overexpressed in chimeric plants, with *GmRIC1* achieving complete suppression of nodulation in wild-type plants. In contrast, *GmNIC1* appears to act locally (as expected for nitrate inhibition of nodulation; see Carroll and Gresshoff, 1983) and only partially, but significantly, suppresses nodulation when overexpressed (Reid *et al.*, 2011a). The nodulation suppression activity (NSA) of *GmRIC1/GmRIC2* is epistatically suppressed in a *GmNARK*-deficient plant (Reid *et al.*, 2011a), suggesting that the peptide signals through the LRR-RK (reminiscent of the CLV3–CLV1 situation).

The systemic activity of these CLE peptides, and the shoot involvement through *GmNARK*, suggests that they may act as long-distance signal ligands for *GmNARK* in the shoot [NB: similar NSA was detected for CLE overexpression in *Lotus japonicus* (Okamoto *et al.*, 2009) and *Medicago truncatula* (Mortier *et al.*, 2010)]. Indeed, it is predicted that perception of *GmRIC1/GmRIC2* by *GmNARK* in the shoot leads to the production of a novel shoot-derived inhibitor (SDI) signal that is transported to the root where it inhibits further nodulation events (Lin *et al.*, 2010, 2011), thus completing the systemic loop of AON (reviewed in Reid *et al.*, 2011b). Knock-down of the two related CLE genes in *M. truncatula* resulted in an increase in nodule numbers, further indicating their likely role in inducing AON (Mortier *et al.*, 2012). Despite this evidence, efforts to detect CLE peptides in xylem sap samples or root tissues of *GmRIC1*-overexpressing plants have not identified CLE peptides to date (Reid *et al.*, 2012).

Genes encoding CLE peptides are widely distributed in plant species, including >20 CLE peptide encoding genes in *Arabidopsis* and over at least 40 in soybean (Cock and McCormick, 2001; Oelkers *et al.*, 2008; Mortier *et al.*, 2011). CLE peptides possess three basic domains, namely an N-terminal transit (signal) peptide, a variable region which does not share a high degree of homology within the family (beyond predicted hydrophobicity), and a 12–13 amino acid CLE domain (Cock and McCormick 2001). Post-translational modifications and processing are required to produce the 12–13 amino acid mature CLE peptide (Kondo *et al.*, 2006; Ohyama *et al.*, 2009). These modifications include proline hydroxylation and arabinosylation which have been detected for several CLE peptides [CLV3 and CLE2 (Ohyama *et al.*, 2009), CLE9 (Shinohara *et al.*, 2012)]. Amino acid residues neighbouring the CLE domain have been proposed to be required for efficient processing of the mature peptide through proteolytic cleavage (Ni and Clark, 2006; Djordjevic *et al.*, 2011; Ni *et al.*, 2011). The transit peptide has been shown to be critical to the nodule inhibition and secretion of green fluorescent protein (GFP)-tagged *GmRIC1* and *GmRIC2* peptides (Lim *et al.*, 2011). In addition to the conserved CLE peptide domain, several of the rhizobia-induced CLE peptides in *L. japonicus*, *M. truncatula*, and *Glycine max*, as well as *AtCLV3*, possess a conserved extension in the C-terminal region of the CLE pre-propeptide comprising dual proline residues. The functional significance of these residues is unclear, but they may assist in processing, protection from degradation, or binding of the CLE peptide.

A number of studies using *AtCLV3* have employed site-directed mutagenesis (SDM) constructs *in vivo* (Ni *et al.*, 2011; Song *et al.*, 2012), and *in vitro* peptide application (Kondo *et al.*, 2008), or domain swap techniques (Meng *et al.*, 2010), to decipher the role of each of the domains of the CLE pre-propeptides. Alanine replacement mutants at each of the residues within the *AtCLV3* domain and surrounding residues have shown that mutants at the R1, P4, G6, D8, P9, L10, H11, and H12 residues of the *AtCLV3* peptide were significantly compromised in their ability to complement *Atclv3* mutants (Song *et al.*, 2012). Synthetic CLE peptides consisting solely of the CLE domain residues, and engineered genes encoding the transit peptide directly adjacent to the CLE domain, have also been shown to be biologically active and effective at rescuing some *Atclv3* phenotypes (Fiers *et al.*, 2005, 2006). In addition to the importance of the CLE domain, the R70 residue of *AtCLV3*, which immediately precedes the CLE domain, has been identified as a peptidolytic processing site; however, mutants at this residue continue to maintain activity (Ni and Clarke, 2006). Up to four residues may be required for cleavage recognition and processing at this residue in the N-terminus of the CLE peptide. A serine protease is likely to be the precise cleaving agent at this location, whereas a progressive carboxypeptidase acts at the C-terminus (Ni *et al.*, 2011). Mutations at the EE residues preceding the *AtCLV3* peptide domain blocked processing but continued to rescue mutant phenotypes, which may indicate that the processing is not required to be highly efficient due to high specificity and very low concentrations of the peptide required for activity.

It has been shown that the CLE domain and transit peptide determines the function and specificity of the peptide (Fiers *et al.*, 2006; Meng *et al.*, 2010), probably through determining to which tissues and receptors the ligand is presented. This tissue specificity is also a result of the diverse expression patterns that exist for the CLE-encoding genes (Sharma *et al.*, 2003; Jun *et al.*, 2010).

The *GmRIC1* CLE domain shares high sequence similarity with the *Arabidopsis* CLE1–CLE7 peptides, with the consensus sequence only varying at the final residue (H in the *Arabidopsis* CLE1–CLE7 peptides compared with N in the nodulation CLE peptides). The function of these *Arabidopsis* CLE peptides remains to be determined; they do not possess the common root apical meristem (RAM)-arresting phenotypes of other CLE peptides and do not induce significant changes in SAM size when applied directly to the plant (Kinoshita *et al.*, 2007). It should be noted that direct plant application may lessen activity because of degradation and possible uptake barriers. Overexpression of genes encoding *AtCLE1–AtCLE7* peptides does, however, indicate that they possess biological activity as it results in phenotypes similar to *AtCLV3* overexpression, albeit with less severe phenotypes (Strabala *et al.*, 2006). Key residues within *GmRIC1* are conserved throughout CLE peptides, including an almost universal conservation of the residues indicated in bold on the *GmRIC1* sequence: **RLAPEGDPHHN** (italics indicate one of two possible amino acids; Oelkers *et al.*, 2008).

The complete suppression phenotype obtained in *GmRIC1*-overexpressing roots of soybean (Reid *et al.*, 2011a) provides an excellent system to investigate the contribution of key residues within the CLE peptide to its overall function. To investigate the relative importance of each residue in the CLE domain to the function of *GmRIC1*, an SDM study was initiated. In addition, chimeric constructs comprised of swapped functional domains of *GmRIC1* and *GmNIC1* were generated to identify which domains contribute to nodule suppression activity and which are critical for localization of the peptide. These experiments allowed the identification of the key residues required for nodule suppression in soybean and will assist in the identification of biologically active CLE peptides capable of nodule suppression in other legume species. These experiments also provide a basis to compare the function of individual residues within nodulation-suppressing CLE peptides with those identified as critical in different species and developmental processes such as obtained from the study of *AtCLV3*.

Materials and methods

General plant and bacterial growth conditions and the nodulation assay

Soybean (*Glycine max* L. Merrill) wild-type Bragg plants were grown in controlled glasshouse conditions (28 °C/26 °C day/night) in 4 litre pots containing grade 3 vermiculite. Plants were watered as required with a modified nutrient solution lacking nitrogen (Herridge, 1982). Plants were inoculated with ~200 ml of *Bradyrhizobium japonicum* CB1809 grown in yeast-mannitol broth (YMB) at 28 °C for 4 d and diluted to ~OD₆₀₀ 0.01. Nodulation was scored 3 weeks after inoculation. Student's *t*-tests were used to determine statistical differences.

Generating constructs and transgenic soybean roots

Constructs bearing the coding sequence of *GmRIC1* and *GmNIC1* cloned in the sense direction only of the pKANNIBAL vector for expression of RNA interference (RNAi) constructs were used as described previously (Wesley *et al.*, 2001; Reid *et al.*, 2011a). *Pfu* polymerase (Stratagene, La Jolla, CA, USA) was used to amplify PCR products incorporating restriction nuclease sites from these constructs. *XhoI/EcoRI* (for) and *KpnI* (rev) 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 available at *JXB* online). Likely clones were confirmed by direct DNA sequencing and capillary separation. Constructs were subcloned into p15SRK2 integration vectors (A. Kereszt, unpublished) as a *NotI* fragment before tri-parental mating to introduce them into *Agrobacterium rhizogenes* K599. *Agrobacterium rhizogenes* was subsequently used for the induction of transgenic soybean hairy roots according to Kereszt *et al.* (2007) and Lin *et al.* (2011).

Site-directed mutagenesis and domain swaps

Point mutants of *GmRIC1* were introduced into the cloning vector pKANNIBAL:*GmRIC1* using PCR-based SDM. The forward and reverse oligonucleotide primers (Supplementary Table S1 at *JXB* online) were complementary to the opposite strands of the vector, and contained the desired mutation in the middle of the primers. Extension with these primers generated mutated plasmids at the relevant sites. Each PCR had a total volume of 25 µl containing 5 µl of 5× KAPA HiFi Buffer, 0.75 µl of 10 mM dNTPs, 1 µl of each primer (10 µM), 0.25 µl of 25 mM MgCl₂, 0.5 µl of KAPA HiFi Polymerase (KAPA biosystems, cat. kk2101), and 6 ng of pKANNIBAL:*GmRIC1* DNA. The reaction program was as follows: one cycle of initial denaturation (95 °C for 3 min 30 s), three cycles of amplification with a lower annealing temperature (95 °C for 20 s, 60 °C for 15 s, 72 °C for 3 min 30 s), 19 cycles of amplification with a high annealing temperature (65 °C), and one cycle of extension (72 °C for 10 min), before the reaction was cooled to 4 °C. The PCR products were treated with the methylation-sensitive endonuclease *DpnI* (NEB, Ipswich, MA, USA) for 1 h at 37 °C to digest the parental DNA template and select for mutation-containing plasmids. The resulting undigested plasmids were used to transform competent TOP10 cells. All constructs were confirmed by direct DNA sequencing.

Chimeric gene domain swaps between regions of *GmRIC1* and *GmNIC1* were derived from pKANNIBAL:*GmRIC1* and pKANNIBAL:*GmNIC1*. This required using either a long reverse primer method for modifications directly at the C-terminus (Meng *et al.*, 2010) or the generation of overlapping PCR products before amplification with the outermost forward and reverse primers (Heckman and Pease, 2007). Primer sequences are shown in Supplementary Table S2 at *JXB* online. For the purpose of constructing chimeric genes, the transit peptide was identified through SignalP prediction of the most likely cleavage sites (Bendtsen *et al.*, 2004) and comprises residues 1–29 for *GmRIC1* and 1–30 for *GmNIC1*. The variable region comprises the central residues from 30 to 76 inclusive in *GmRIC1* and 31 to 75 in *GmNIC1*, while the CLE domain comprised the 12 amino acids from 77 to 88 in *GmRIC1* and 76 to 87 in *GmNIC1*. The residues 89–95 in the C-terminal region of *GmRIC1* were defined as the C-terminal extension.

Results

Identification of critical *GmRIC1* residues for nodule inhibition

To investigate the functional importance of the amino acids within the CLE domain of *GmRIC1*, point mutations were

produced for each residue (Fig. 1A). The *GmRIC1* CLE domain is comprised of 12 amino acids positioned from R77 to N88 in the *GmRIC1* sequence, which corresponds to R1–N12 in the CLE domain. Alanine substitutions were created at the L2, E5, G6, P7, D8, P9, H10, H11, and N12 residues,

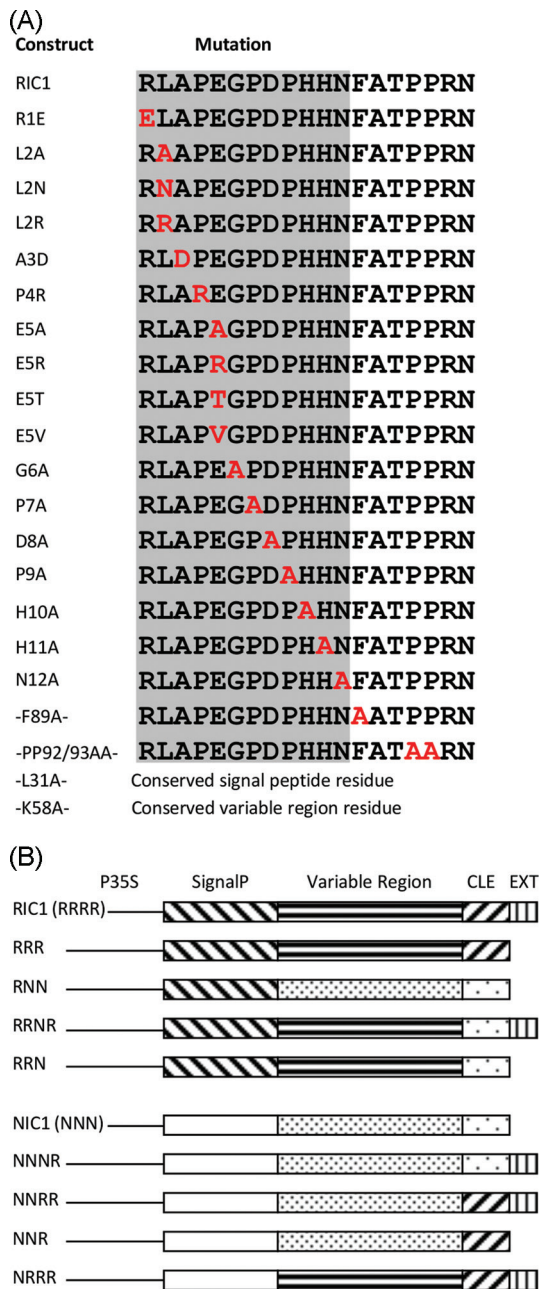


Fig. 1. Constructs used in the nodule suppression assay. (A) Site-directed mutants derived from *GmRIC1* were created for each residue in the CLE domain as well as several well-conserved residues outside the CLE domain. (B) Chimeric gene constructs were derived from combinations of the *GmRIC1* and *GmNIC1* signal peptide, variable region, CLE domain, and C-terminal extension. Each construct was named according to the source of each domain (e.g. RRRR for *GmRIC1*) and was expressed under the 35S promoter in *Agrobacterium rhizogenes*-induced hairy roots. (This figure is available in colour at JXB online.)

and additional substitutions were also made at R1E, A3D, and P4R. All mutants were derived from a *GmRIC1* overexpression construct previously shown to inhibit soybean nodulation completely in a systemic and *GmNARK*-dependent manner (Reid *et al.*, 2011a).

The nodule suppression activity of each construct was determined by comparing nodule numbers on *A. rhizogenes*-induced hairy roots of the empty vector (114 nodules \pm 10, $n=12$ in one representative experiment of four), with *GmRIC1* overexpression constructs showing 100% suppression of nodulation (0 nodules \pm 0, $n=12$ in each of four replicates). Constructs bearing A substitutions at L2, E5, P9, and H10 retained complete nodule suppression activity, while the A3D mutant showed only a slight disruption in suppression (93% suppression of nodulation, $P < 0.05$ using Student's *t*-test; Fig. 2). R1E (72%), E5R (69%), G6A (52%), H11A (41%), and P7A (40%) all showed an intermediate loss of suppression, while the most severe loss of suppression was observed in N12A (21%), P4R (29%), and D8A (18%) when compared with RIC1 ($P < 0.05$).

Generation of multiple variants at conserved residues to investigate the effect of amino acid charge and size on *GmRIC1* function

To determine the effect of amino acid charge and size on the function of the *GmRIC1* peptide, multiple substitutions were produced at two conserved residues in the CLE domain, L2 and E5 (Fig. 3). A, N, and R mutants were created at L2, while A, T, V, and R mutants were created at E5 (Figs 1A, 3). Each of the three mutations at L2, and A, T, and V mutations at E5 maintained 100% suppression of nodulation. In contrast, the E5R substitution resulted in 69% suppression of nodulation ($P < 0.05$, Fig. 2).

Mutation of residues outside the *GmRIC1* CLE domain to determine their role in nodule inhibition

Certain residues located outside of the CLE domain are thought to be critical for the production of the mature peptide, including the signal peptide, the C-terminal region, and potential cleavage sites within the variable domain (Fiers *et al.*, 2006; Ni and Clark 2006; Djordjevic *et al.*, 2011; Ni *et al.*, 2011). Mutations were produced at several of these well-conserved residues to determine their effect on *GmRIC1* activity. Alanine substitutions were made at both the L31 and K58 residues of *GmRIC1*. L31 is located adjacent to the C-terminus of the signal peptide and shares 100% identity across the known nodulation CLE peptides of *L. japonicus*, *M. truncatula*, and *G. max*. K58 is the best conserved residue in the variable region of the nodulation CLE peptides (70% identity between known nodulation CLE peptides). Neither substitution resulted in a reduction in suppression activity of *GmRIC1* (Fig. 2).

Alanine substitutions were also produced at two locations in the C-terminal region of *GmRIC1*, including the residue immediately adjacent to the predicted mature peptide (F89A), and two conserved P residues (92/93, a single construct bearing an AA substitution), which are conserved between 70%

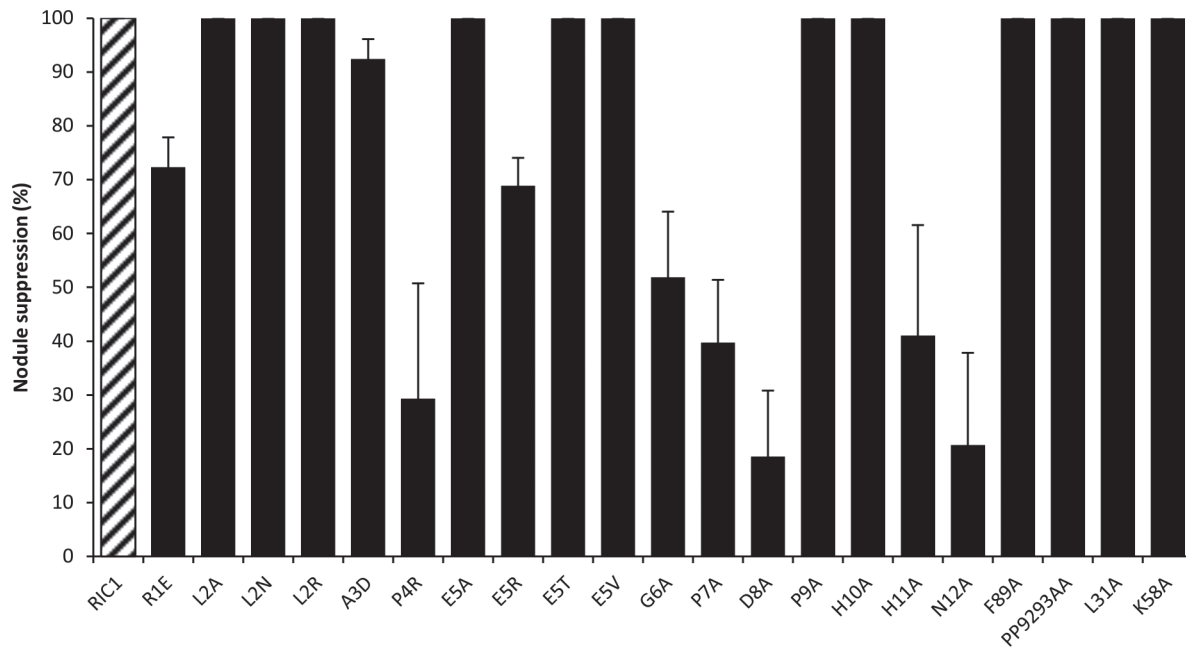


Fig. 2. Nodulation suppression activity (NSA) in *GmRIC1* site-directed mutants. Suppression of nodulation obtained by overexpression of each of the site-directed mutants was determined relative to the nodule number on empty vector-transformed controls. *GmRIC1* and mutants that do not disrupt *GmRIC1* function exhibit complete suppression.

of nodulation-related CLE peptides. These substitutions also maintained complete nodulation suppression activity.

Amino acid conservation in CLE peptides in relation to functional importance

To determine whether amino acid conservation is a good indicator of functional importance, the results were compared

with multiple sequence alignments of all CLE peptides reported to possess nodulation suppression activity (Reid *et al.*, 2011a) and with recent *AtCLV3* experiments involving site-directed mutagenesis (Song *et al.*, 2012). Within the nodulation-related CLE peptides (Fig. 4), R1, P4, G6, P7, and H11 residues show complete conservation and were all critical to activity in the present study. Additionally, the D8 and N12 residues are conserved in six out of seven nodulation-suppressing

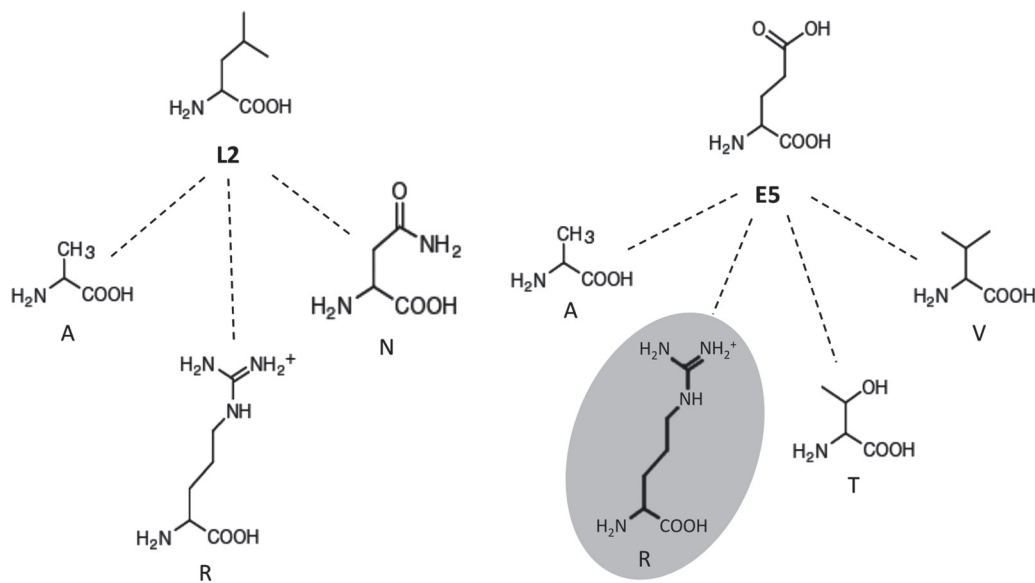


Fig. 3. Site-directed mutants of *GmRIC1* to determine effects of charge and size of amino acid substitutions. Multiple mutations were produced at two well-conserved residues within the CLE domain, L2 and E5. A, R, and N residues were substituted for L2, while A, R, T, and V were substituted for E5. The shaded R residue represents the only mutation that disrupted the complete suppression of nodulation.



Fig. 4. Amino acid conservation and importance to GmRIC1 activity. Consensus sequence and level of conservation of each residue in the CLE peptides known to inhibit nodulation are indicated by LOGO alignment (Crooks et al., 2004). Relative conservation of the mutated GmRIC1 residues with reduced (blue) or wild-type (red) nodule suppression activity is shown. The blue E residue represents the activity of the E5R mutation, which most significantly altered activity.

CLE peptides and showed reduced suppression activity when mutated. The E5 residue of *GmRIC1* differs from the consensus (G in 5/7) in other nodulation CLE peptides and did not alter activity when mutated to A, T, or V, but was compromised by a mutation to R (E81R). Similarly, the *GmRIC1* A3 residue differed from the consensus sequence at that position (S in 5/7) and had only a small effect on suppressive activity when mutated. The *GmRIC1* L2 residue is completely conserved between the nodulation-suppressing CLE peptides; however, three mutations to A, N, or R indicated that it was not critical to suppression. Neither P9, which is conserved in 70% of nodulation CLE peptides, nor H10, which differs from the consensus (Q in 4/7), was found to alter *GmRIC1* activity. None of the alanine mutations in conserved residues outside of the CLE domain, which included L31 (100% conserved), K58 (5/7), and PP92/93 (5/7), was shown to alter activity.

Comparison of *GmRIC1* with *AtCLV3* showed that seven out of 12 residues in the CLE domain are common between them (see Table 1). Two of these seven conserved residues (P7 and P9) did not share functional importance between the present results and those of Song et al. (2012). The P7 residue was critical to *GmRIC1* but not to *AtCLV3* activity, whereas the opposite was true at P9, which was critical to *AtCLV3* but not to *GmRIC1*. One of the four non-conserved residues (CLV3-H12/*RIC1*-N12) was critical to the function of both *Atclv3* complementation and legume suppression of nodulation.

Contribution of functional domains to *GmRIC1* and *GmNIC1* activity

In contrast to *GmRIC1* overexpression, which results in 100% suppression of soybean nodulation, overexpression of

Table 1. Comparison of critical residues in *GmRIC1* and *AtCLV3* as determined by site-directed mutagenesis by Song et al. (2012).

<i>GmRIC1</i> peptide	R	L	A	P	E	G	P	D	P	H	H	N
<i>AtCLV3</i> peptide	R	T	V	P	S	G	P	D	P	L	H	H
Residue required for <i>GmRIC1</i> nodule suppression	Y	N	*	Y	**	Y	Y	Y	N	N	Y	Y
Residue required for complementation of <i>clv3</i> fifth whorl ovary phenotype	Y	N	N	Y	N	Y	*	Y	Y	*	Y	Y

*Minor change; **only for E>R change.

GmNIC1 only results in an ~50% reduction in nodule numbers (Reid et al., 2011a). To determine which domains contribute to this difference in suppression, several constructs were produced encoding chimeric versions of these two peptides (Fig. 1B). The domains investigated included the transit peptide, variable region, CLE domain, and C-terminal extension. The constructs were named according to the source of their domain. For example, an unmodified *GmRIC1* construct was named RRRR, whereas a construct bearing the *GmRIC1* signal peptide, *GmNIC1* variable region, and CLE domain, and lacking the C-terminal extension, was named RNN, noting that the native *GmNIC1* peptide lacks the C-terminal extension domain (Fig. 1B).

Analysis of the role of the signal peptide domain in *GmRIC1* and *GmNIC1* activity

As the signal peptide has been shown to control *GmRIC1* secretion (Lim et al., 2011), experiments were conducted to determine whether the *GmNIC1* signal peptide was functionally interchangeable with *GmRIC1*. A *GmRIC1* construct was produced where the *GmNIC1* signal peptide replaced the native signal peptide (NRRR). The equivalent *GmNIC1* construct bearing the native *GmRIC1* signal peptide (RNN) was also produced. The RNN construct resulted in significantly increased suppression of nodulation relative to *GmNIC1* ($P < 0.001$; Fig. 5). In contrast, the NRRR construct showed a significantly lower level of suppression relative to the complete suppression by the *GmRIC1* peptide (24% suppression, $P < 0.001$).

Analysis of the role of the C-terminal extension domain in *GmRIC1* and *GmNIC1* activity

GmRIC1 encodes a C-terminal extension after the CLE peptide domain which is conserved in 70% of nodulation-related CLE peptides as well as in *AtCLV3*, but is absent in *GmNIC1*. To determine if this domain plays a critical role in nodule suppression activity, both a *GmRIC1* construct where this domain was deleted (RRR) and a *GmNIC1* construct where the C-terminal domain of *GmRIC1* was added (NNNR) were produced. The function of *GmRIC1* was not altered by the deletion of the C-terminal extension (RRR) as complete nodule suppression was maintained (Fig. 5). In contrast, the addition of the *GmRIC1* C-terminal extension to *GmNIC1* (NNNR) resulted in a significant increase in the suppression of nodulation (90% suppression, $P < 0.001$).

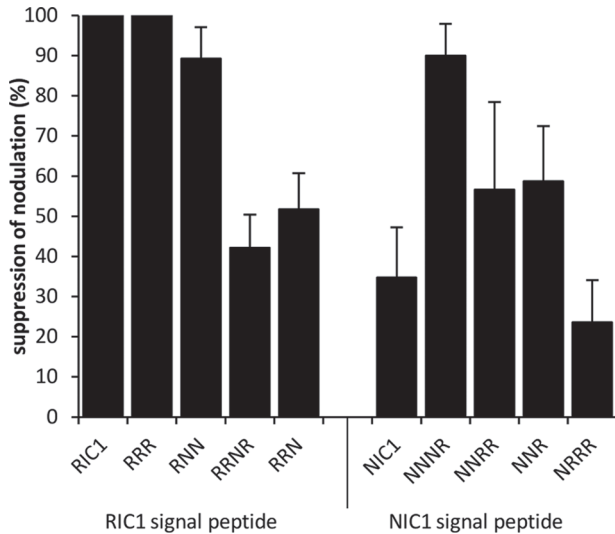


Fig. 5. Suppression of nodulation by chimeric *GmNIC1* and *GmRIC1* constructs. Chimeric gene constructs were compared with the suppression of nodulation obtained by *GmRIC1* and *GmNIC1* overexpression. Genes were named according to the source of the transit peptide, variable region, CLE domain, and C-terminal extension, respectively (*GmRIC1* would be named RRRR, while *GmNIC1* is NNN as it lacks the C-terminal extension).

Analysis of the role of the CLE peptide domain in *GmRIC1* and *GmNIC1* activity

Constructs were created that swapped the CLE domains of *GmRIC1* and *GmNIC1* (RRNR and NNR) to determine their contribution to the suppression of nodulation. Constructs that swapped the CLE domain regions in the context of the C-terminal region of the donor peptide [the C-terminal extension of *GmRIC1* (NNRR) or the stop codon for *GmNIC1* (RRN)] were also produced. The *GmNIC1* peptide bearing the *GmRIC1* CLE domain (NNR) showed no significant change in activity relative to the native *GmNIC1* peptide ($P=0.11$; Fig. 5). Similarly, the construct comprising the *GmNIC1* transit peptide and variable region together with *GmRIC1* CLE domain in native context adjacent to the *GmRIC1* C-terminal extension (NNRR) did not significantly alter suppression activity from that of *GmNIC1*. Reciprocal swaps, where *GmRIC1* contained the *GmNIC1* CLE domain (RRNR), showed significantly reduced nodule suppression activity relative to *GmRIC1* ($P < 0.001$; Fig. 5). Likewise, the *GmRIC1*-based construct carrying the CLE domain of *GmNIC1* but lacking a C-terminal extension (RRN) showed a significant reduction in suppression activity relative to both native *GmRIC1* and the *GmRIC1* construct lacking the C-terminal extension (RRR) ($P < 0.001$; Fig. 5).

Analysis of the role of the variable region domain in *GmRIC1* and *GmNIC1* activity

The central variable region of CLE peptides is not highly conserved amongst the nodulation-related CLE peptides and has not been unequivocally shown to exert functional

specificity in previous CLE domain studies (Ni and Clark, 2006; Meng *et al.*, 2010). Accordingly, specific constructs with direct swaps of this region were not produced. However, to determine any contribution of the variable region to differences in suppression activity, combinations of the constructs that varied only in this region (NNRR versus NRRR and RRN versus RNN) were compared. No significant difference in suppression activity was observed between the two alternative variable domains in the constructs with *GmNIC1* transit peptide and the *GmRIC1* CLE domain (NNRR versus NRRR; $P=0.16$; Fig. 5). In contrast, the *GmNIC1* variable region enhanced suppression activity when a *GmRIC1* transit peptide and *GmNIC1* CLE domain were present adjacent to the variable region (RNN versus RRN; $P < 0.005$; Fig. 5).

Discussion

Although *GmNARK* and *AtCLV1* are closely related homologues with high sequence similarity, little is known about their ligand specificity. The functional importance of the domains and many individual amino acids within *AtCLV3* has been well studied and provides an excellent basis to identify functional divergence from *GmRIC1* (Kondo *et al.*, 2008; Ni *et al.*, 2011; Song *et al.*, 2012). The experimental system used here, which was employed to identify critical residues within the *GmRIC1* peptide through assessment of nodule inhibition, provided a specific and easy to quantify assay having a large and dynamic range of detectable nodule numbers (0 in *GmRIC1* compared with an average of 114 nodules in controls). In addition, the chimeric *GmRIC1*-overexpressing plants do not show any secondary root or shoot developmental phenotypes outside of nodulation. This is not the case for all nodulation-related CLE peptides, as the application or expression of *MtCLE12* inhibits root growth in addition to nodulation (Saur *et al.*, 2011). These differences make the use of *GmRIC1* in the present assay ideal for analysing the function of CLE peptides in suppressing nodulation.

It was found that the R1, P4, G6, P7, D8, H11, and N12 residues within the *GmRIC1* CLE domain were most important to nodule suppression activity. In contrast, mutation of the A3 residue provided only a small disruption of suppression, while mutations in the L2, P9, and H10 residues did not alter activity, suggesting that they play a relatively minor role. The E5 residue was only disrupted by mutation to R, while A, T, and V mutants at E5 were unaffected in nodule suppression activity. Outside of the CLE domain, none of the individual residues tested was found to be critical; however, the *GmRIC1* signal peptide, CLE domain, and C-terminal extension region were all found to enhance the activity of *GmNIC1*.

AtCLV3 and *GmRIC1* CLE domain sequences are well conserved, including perfect conservation at seven out of 12 residues. Complementation of *Atclv3* plants with alanine substitution mutants showed that the R1, P4, G6, D8, P9, L10, H11, and H12 residues within the CLE domain were all critical to obtaining full complementation, whereas T2, V3, S5, and P7 mutants showed no alteration in activity (Song

et al., 2012). Interestingly, the P7 residue which has been identified as a site of post-translational modification (Ohshima *et al.*, 2009) was not critical to function. Of the four residues which were identified as unimportant in *AtCLV3*, three showed either no or only small loss of suppression activity in the present experiments and none of these was conserved (L2, A3, and E5). The fourth residue found to be unimportant in *AtCLV3*, P7, was shown to be critical to activity in the present study. The structural effects of these changes and why they show increased tolerance of mutation remains unknown at this stage, but may be further clarified through determination of receptor–ligand structures and binding studies.

Interestingly, the *GmRIC1* P9 residue was not identified as important in the present study, despite being critical to *AtCLV3* activity (Kondo *et al.*, 2011; Song *et al.*, 2012). Indeed, using peptoid replacement of proline residues, Kondo *et al.* (2011) showed that substitution could alter activity without altering binding. This difference may indicate an alteration of the post-translational modification site between *GmRIC1* and *AtCLV3*, or a selective preference in the LRR receptor. The *GmRIC1* N12/*AtCLV3* H12 residue was shown to be critical to both peptides despite the lack of conservation (although both fall into the same charge class). It should be noted that both H and N residues are common at the C-terminus of CLE peptides (Oelkers *et al.*, 2008). The identification of D8 as a critical residue also confirms the finding by Song *et al.* (2012) of the importance of this residue despite it being identified through *in vitro* application studies as lacking functional importance (Kondo *et al.*, 2008). In contrast to this, the present finding that the H10 residue of *GmRIC1* did not alter activity was different from the minor role identified through *in vivo* complementation studies using *Atclv3* mutants (Song *et al.*, 2012), but was consistent with a lack of activity observed at this position from *in vitro* peptide application studies (Kondo *et al.*, 2008).

Whether differences in amino acid residue charge and size could affect *GmRIC1* activity at two residues that were initially identified as showing no effect when substituted with alanine (L2 and E5) was investigated. Loss of function by the E5R mutation suggests that the chemical properties of this amino acid do play a role, as only this acidic to basic change altered the suppression activity. This indicates that selection at these residues remains important, albeit with a greater tolerance to changes. It is possible that many of the residues determined to be unimportant are indeed critical to activity simply due to their role in establishing the peptide backbone.

Despite high levels of conservation at several amino acids located outside of the CLE domain, none showed a loss of activity in our study using *GmRIC1*. This is consistent with previous studies (Ni *et al.*, 2011; Song *et al.*, 2012) that failed to identify residues critical to activity outside of the CLE domain, despite several being identified as likely protease cleavage sites. This may indicate that a consensus sequence or motif is sufficient to target protease cleavage, or that sequence within the CLE peptide domain itself directs cleavage of surrounding residues.

Irrespective of the lack of critical residues identified outside of the CLE domain, the domain swap results provided

significant evidence for the importance of these domains to the activity of nodulation-related CLE peptides. The importance of the *GmRIC1* transit peptide was confirmed to confer a significant functional advantage over the equivalent sequence from *GmNIC1*. This is consistent with previous results identifying the transit peptide as important for the secretion of *GmRIC1* (Lim *et al.*, 2011). Several conserved residues within the transit peptide of nodulation-related peptides of soybean, *L. japonicus*, and *M. truncatula* were identified as altered in *GmNIC1* (Reid *et al.*, 2011a) and it seems possible that some of these residues could play a role in the localization of the peptide.

Like the transit peptide domain, the CLE domain of *GmRIC1* was functionally superior relative to that of *GmNIC1*. *GmNIC1* is the only nodulation-related CLE peptide with a H residue at position 12, where all others possess N12. Our studies demonstrated that N12 is critical to *GmRIC1* suppression of nodulation. Interestingly, *GmNIC1* is also one of only two identified nodulation-related CLE peptides lacking P9, despite alanine substitutions at this residue maintaining complete activity in the present study. Despite the conservation of two proline residues within the C-terminal tail region of *GmRIC1* and other nodulation-related CLE peptides, no functional importance could be determined for the region in *GmRIC1* as constructs with the domain deleted retained full activity. Interestingly, however, the domain did confer some advantage to *GmNIC1* when it was added. This suggests that the domain possibly enhances processing or protects the mature CLE from breakdown but is not essential to binding. The unchanged activity of the *GmRIC1* constructs may suggest that overexpression of *GmRIC1* generates a large enough pool of the mature peptide such that protection from cleavage is less important, or can be overcome by saturation of proteases with the peptide. In *GmNIC1*, reduced breakdown of the mature peptide owing to the C-terminal extension in the NNNR construct may increase the lifetime and therefore the activity of the resulting peptide.

The least conserved region within CLE peptides is the region between the signal peptide and the CLE domain, termed the variable domain. The present results showed that neither the *GmRIC1* nor *GmNIC1* variable regions conferred a consistent advantage over the other. Of the two comparisons which were made, the *GmNIC1* variable region did increase suppression in one case, although it did not restore the activity to control levels and a comparable change was not observed in the reciprocal construct. This probably indicates that some advantage is conferred by different variable regions, especially if a specific protease is involved in cleavage, but that processing is likely to be by a less specific protease or is directed by neighbouring domains.

Taken together, the domain-swap results indicate a significant advantage of the *GmRIC1* transit peptide, CLE domain, and, to a lesser extent, the C-terminal tail region, over the corresponding domains in *GmNIC1*. Although *GmNIC1* does inhibit nodulation in a *GmNARK*-dependent manner, the low efficiency of this suppression may indicate that it is not the only CLE domain peptide in soybean that responds to nitrate, or that other mechanisms that are not dependent

on CLE peptide–NARK signalling are more significant players. The data obtained in these studies may provide valuable assistance in identifying possible additional nitrate-responsive CLE peptides with increased nodule suppression efficiency. The low efficiency of *GmNIC1* may also indicate that *GmNIC1* has a secondary function in another nitrate-dependent signalling pathway via an alternative receptor. The lack of secondary phenotypes resulting in *GmNIC1*-overexpressing plants, however, indicates that the developmental changes are subtle or are in some way altered in *Agrobacterium*-induced hairy root systems.

As one of only a few studies to investigate CLE peptide function outside of *Arabidopsis* and rice, the present results provide useful insight into the functional importance of the protein domains and many individual amino acids of this group of peptides. For example, the critical importance of the *GmRIC1* transit peptide identified here may provide a basis for further analysis of this domain to determine key residues for its activity. By identifying the tolerance to mutation at each of the residues of the RIC1 CLE domain and identifying the residues which are critical only in nodulation and not SAM regulation, the findings also provide useful insight into the evolution of CLE peptide signalling in plant development. The results may assist in the identification of CLE peptides in other legumes, particularly those that are likely to possess nodule inhibitory activity. Indeed, the highly sensitive and robust nodule suppression assay used here provides an excellent tool for future nodule suppression studies.

Supplementary data

Supplementary data are available at *JXB* online.

[Table S1](#). Primer sequences for site-directed mutagenesis.

[Table S2](#). Primers for domain swap constructs.

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