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Goldacre Review

The structure and activity of nodulation-suppressing CLE peptide hormones of legumes

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Abstract. Legumes form a highly-regulated symbiotic relationship with specific soil bacteria known as rhizobia. This interaction results in the *de novo* formation of root organs called nodules, in which the rhizobia fix atmospheric di-nitrogen (N_2) for the plant. Molecular mechanisms that regulate the nodulation process include the systemic 'autoregulation of nodulation' and the local nitrogen-regulation of nodulation pathways. Both pathways are mediated by novel peptide hormones called CLAVATA/ESR-related (CLE) peptides that act to suppress nodulation via negative feedback loops. The mature peptides are 12–13 amino acids in length and are post-translationally modified from the *C*-terminus of tripartite-domain prepropeptides. Structural redundancy between the prepropeptides exists; however, variations in external stimuli, timing of expression, tissue specificity and presence or absence of key functional domains enables them to act in a specific manner. To date, nodulation-regulating CLE peptides have been identified in *Glycine max* (L.) Merr., *Medicago truncatula* Gaertn., *Lotus japonicus* (Regel) K.Larsen and *Phaseolus vulgaris* L. One of the *L. japonicus* peptides, called LjCLE-RS2, has been structurally characterised and found to be an arabinosylated glycopeptide. All of the known nodulation CLE peptides act via an orthologous leucine rich repeat (LRR) receptor kinase. Perception of the peptide results in the production of a novel, unidentified inhibitor signal that acts to suppress further nodulation events. Here, we contrast and compare the various nodulation-suppressing CLE peptides of legumes.

Additional keywords: autoregulation of nodulation, legume nodulation, nitrate-regulation of nodulation, nodule, plant peptide signalling, symbiosis.

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Introduction

The common agricultural practice of using nitrogen-based fertilisers to increase crop yields has been highly successful in generating sufficient food for the world's ever-growing population. It has been a major part of the 'green revolution' instigated more than 50 years ago. However, adverse economical and ecological consequences are beginning to outweigh the benefits of nitrogen fertiliser use (Erisman *et al.* 2008; Sutton *et al.* 2011; Jensen *et al.* 2012).

Symbiotic nitrogen fixation represents an alternative to chemical nitrogen fertiliser use. It involves a relationship mainly formed between plant species of the family Fabaceae, commonly known as legumes, and soil bacteria, collectively referred to as rhizobia. Major legume crop and pasture species include soybean, pea, common bean, clover, cowpea, medic, chickpea, lentil and peanut. Biological nitrogen fixation from this legume–rhizobia relationship currently results in ~50–70 Tg of nitrogen added into global agricultural systems each year (Herridge *et al.* 2008; Jensen *et al.* 2012).

The legume-rhizobia relationship is signified by the formation of a new plant organ, called the nodule. Nodule development is orchestrated by a complex signalling interaction (Ferguson and Mathesius 2003, 2014; Ferguson *et al.* 2010; Desbrosses and Stougaard 2011; Oldroyd 2013). Once formed, the nodule acts to house the rhizobia that provide the plant with a useable form of reduced nitrogen (namely ammonia) using a specialised enzyme complex to 'fix' un-reactive atmospheric di-nitrogen gas (N_2). In return, the rhizobia are provided with a carbon source derived from photosynthesis, predominately malate (Udvardi *et al.* 1988). In addition to increasing current crop yields, this process is exploited in agriculture to improve the nitrogen content and structure of soils by using legumes as rotation crops (Jensen *et al.* 2012).

Control of legume nodule numbers

Nodulation is costly to the host plant in terms of resources; as a result, the plant has developed both local and systemic mechanisms to control its nodule numbers (Delves *et al.* 1986; Gresshoff and Delves 1986; recently reviewed by Reid *et al.* 2011*b*). Local control mechanisms responding to high soil nitrate directly prevent or delay nodule development (Carroll *et al.* 1985*a*; Reid *et al.* 2011*a*). A systemic control mechanism, called the 'autoregulation of nodulation' (AON), is closely associated with the nitrate regulatory pathway, but is induced

by rhizobia, not nitrate, and acts systemically through the shoot, rather than locally in the root (Kosslak and Bohlool 1984; Delves *et al.* 1986; Gresshoff and Delves 1986; Reid *et al.* 2011*a*, 2011*b*).

The AON process begins with the production of a root-derived signal (Gresshoff and Delves 1986), which is expressed in response to a transcription factor, called NIN, involved in cortical cell division during early nodulation events (Soyano *et al.* 2014). This signal, formerly called 'Q' (Gresshoff and Delves 1986), is now known to be a CLAVATA/Embryo surrounding region (ESR) related (CLE) peptide. To date, CLE peptide-encoding genes having a role in nodulation have been identified in *Glycine max* (L.) Merr. (soybean), *Medicago truncatula* Gaertn., *Lotus japonicus* (Regel) K.Larsen and *Phaseolus vulgaris* L. (common bean) (Fig. 1; Okamoto *et al.* 2009, 2013; Mortier *et al.* 2010, 2012; Lim *et al.* 2011; Saur *et al.* 2011; Reid *et al.* 2011*a*, 2013; Ferguson *et al.* 2014). Recent biochemical advances have enabled the isolation and identification of one of the nodulation CLE peptides of *L. japonicus*, called LjCLE-RS2. The mature signal of this CLE peptide is 13 amino acids in length, is derived from a much larger prepropeptide and is post-translationally modified with three β 1-2 linked arabinose moieties at Hyp7 (Okamoto *et al.* 2013).

The nodulation-suppressing CLE peptide signal is exported from the root and transported via the xylem by an unknown mechanism (Okamoto *et al.* 2013) to the leaf phloem parenchyma (Nontachaiyapoom *et al.* 2007) where it is perceived by a leucine-rich repeat serine-threonine receptor kinase (LRR RK), called GmNARK in soybean, LjHAR1 in *L. japonicus*, MtSUNN in *M. truncatula*, PsSYM29 in *Pisum sativum* L. (pea), GsNARK in *Glycine soja* Siebold & Zucc., and PvNARK in common bean (Krusell *et al.* 2002; Nishimura *et al.* 2002; Searle *et al.* 2003; Schnabel *et al.* 2005; Ferguson *et al.* 2014). These LRR RKs may act in a complex with other receptors to perceive the CLE peptide ligand. This includes factors such as LjCLAVATA2/PsCLAVATA2 and LjKLAVIER (Miyazawa *et al.* 2010; Krusell *et al.* 2011). Additional research has identified other factors that may interact with the LRR RK directly, or function downstream of it, to relay the perception of the signal and trigger downstream signalling events. This includes the kinase-associated protein phosphatases, GmKAPP1 and GmKAPP2 (Miyahara *et al.* 2008), the putative ubiquitin fusion degradation protein, GmUFD1a (Reid *et al.* 2012) and the root-acting F-box protein, TOO MUCH LOVE, LjTML (Magori *et al.* 2009; Takahara *et al.* 2013). Following the perception of the nodulation CLE peptide signal, a shoot-derived inhibitor (SDI) signal is produced and transported to the roots, likely via the phloem, to inhibit further nodulation development (Delves *et al.* 1986; Lin *et al.* 2010, 2011; Reid *et al.* 2011*b*; Sasaki *et al.* 2014). Recent studies in *L. japonicus* have indicated a role for cytokinin as a potential SDI-candidate in AON (Sasaki *et al.* 2014).

The gene encoding for the LRR RK is expressed in both shoot and root tissues (Krusell *et al.* 2002; Nishimura *et al.* 2002; Searle *et al.* 2003; Schnabel *et al.* 2005; Nontachaiyapoom *et al.* 2007) and plants having mutations in it exhibit both supernodulation (due to a lack of AON control) and nitrate-tolerant nodulation phenotypes (e.g. Carroll *et al.* 1985*a*, 1985*b*). Grafting studies using soybean have demonstrated that the *GmNARK* LRR RK is required for both AON in the shoot (Delves *et al.* 1986; Reid *et al.* 2011*a*) and nitrate-regulation of nodulation in the root (Reid *et al.* 2011*a*). Similar to AON, the nitrate regulation of nodulation mechanism in soybean begins with the production of a CLE peptide that is predicted to be perceived by GmNARK. However, unlike AON, this CLE peptide, called GmNIC1, responds to nitrate, not rhizobia, and acts locally in the root, not systemically in the shoot. These findings helped to confirm that there are two independent pathways controlling nodulation: the systemic rhizobia-induced AON pathway and the local nitrate-induced regulation of nodulation pathway (reviewed by Reid *et al.* 2011*b*). No candidates for the root-derived inhibitor (RDI) have been identified to date, but as it is produced downstream of GmNARK, it may be similar to, or even the same as, the SDI signal in AON.

Unlike soybean, *L. japonicus* and *M. truncatula* appear to have overlapping local and systemic molecular mechanisms that act to regulate nodulation in response to both rhizobia and nitrate (Okamoto *et al.* 2009; Mortier *et al.* 2010). Although the reason for this difference amongst species is unknown, it is likely that it relates to genomic duplication events undergone in soybean that have enabled genetic divergence and the development of

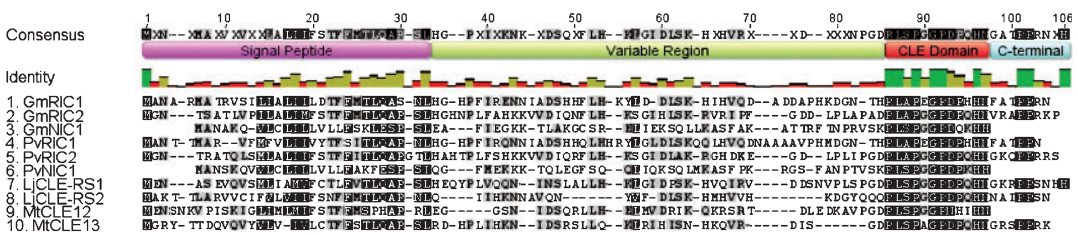

Fig. 1. Multiple sequence alignment and domain structure of the nodulation CLE prepropeptides. Shown are the amino acid sequences of the known nodulation-suppressing CLE peptides of *Glycine max* (soybean), *Phaseolus vulgaris* (common bean), *Lotus japonicus* and *Medicago truncatula*. The alignment was obtained using CLUSTALW multiple alignment (Larkin *et al.* 2007) in Geneious Pro 6.0. Shading of individual amino acids represents conservation amongst the prepropeptides, with the darker the shading the more highly conserved the residue. The CLE domain is highly conserved, with many other conserved residues found in the signal peptide and *C*-terminal extension domains. Conservation is particularly strong between orthologous genes of the different species. Not shown are the homeologous/duplicate copies of the soybean genes, which may have no-, reduced- or an alternative-function.

new molecular signals and mechanisms through the process of neofunctionalisation (Schmutz *et al.* 2010). This may also explain why soybean has three functional CLE peptides that are known to regulate nodule numbers, in addition to three homeologous (duplicate) copies that may have no-, reduced- or diverged-function (Reid *et al.* 2011*a*), whereas *L. japonicus* and *M. truncatula* appear to have only two such peptides (Table 1). Interestingly common bean, which shared a duplication event with soybean 53 million years ago, has orthologous copies of the three soybean CLE peptide genes, but lacks the duplicate copies of each of these genes as a result of not undergoing the more recent genome duplication event approximately 13 million years ago (Ferguson *et al.* 2014).

The CLE peptides that act as a trigger for AON and nitrate-regulation of nodulation belong to a large group of heavily processed, cysteine-poor secreted plant peptides related to AtCLV3 in *Arabidopsis thaliana* (L. Heynh.) (Matsubayashi 2014). AtCLV3 functions in the CLAVATA pathway to regulate the shoot apical meristem stem cell population. It acts as a ligand to a receptor-complex involving AtCLV1, AtCLV2 and AtCORYNE (Ogawa *et al.* 2008). The AtCLV1 receptor is a LRR RK that is highly similar in structure to the LRR RKs that are central to nodulation control. Other *Arabidopsis* CLE peptides of note that are similar to the nodulation CLE peptides of legumes include AtCLE1 to AtCLE7, which have roles in root architecture and development (Cock and McCormick 2001; Strabala *et al.* 2006; Oelkers *et al.* 2008; Araya *et al.* 2014).

The mechanisms controlling nodulation in legumes are highly conserved, as demonstrated by the interspecific function of AON CLE peptides from soybean in common bean and from *M. truncatula* in pea (Osipova *et al.* 2012; Ferguson *et al.* 2014). There are, however, many differences in the sequences, structures and inducing factors of the various nodulation CLE peptides that allow for specificity of function (Fig. 1; Tables 1, 2). These similarities and differences, and how they impact on nodule suppression, are reviewed here.

Key functional domains of CLE peptides

Mature CLE peptide signals are derived from prepropeptides consisting of 3–4 domains: an *N*-terminal signal peptide, a variable region and a CLE domain, with some also having a *C*-terminal extension (Fig. 1). Sequence similarities amongst the nodulation CLE prepropeptides shows the orthologous copies are most similar (Fig. 2; Table 2); however, it is likely that similarities and differences in the individual domains are most critical for driving specificity. Here we discuss the function, conservation and importance of each domain, particularly in respect to their role in the suppression of nodulation.

Signal peptide

The *N*-terminal hydrophobic signal peptide (also referred to as a transit peptide) is widely thought to be responsible for exporting the prepropeptide out of the cell (la Cour *et al.* 2004; Lim *et al.* 2011). It is ~30 amino acids in length and is critical to the specificity of the peptide (Fletcher *et al.* 1999; Reid *et al.* 2013). This domain has a role in exporting the AtCLV3 propeptide into the extracellular space (Rojo *et al.* 2002). A

Table 1. Known nodulation CLE peptides and their key reported features
ND, not determined

Species/peptide	Prepropeptide length (aa)	Receptor	Local/ systemic	Mode of induction	Induction time	Signal peptide motif (TLQAR) conservation	*C*-terminal domain	References
Glycine max								
GmRIC1	95	GmNARK	Systemic	Rhizobia	Early (<12 h)	Y (80%)	Y	Reid *et al.* (2011*a*)
GmRIC2	93	GmNARK	Systemic	Rhizobia	Late (48–72 h)	Y (100%)	Y	Reid *et al.* (2011*a*)
GmNIC1	80	GmNARK	Local	Nitrate		N (<40%)	N	Reid *et al.* (2011*a*)
Phaseolus vulgaris								
PvRIC1	97	PvNARK	ND	Rhizobia	Early (<24 h)	Y (100%)	Y	Ferguson *et al.* (2014)
PvRIC2	93	PvNARK	ND	Rhizobia	Late (<5 days)	Y (100%)	Y	Ferguson *et al.* (2014)
PvNIC1	80	PvNARK	ND	ND	ND	N (<40%)	N	Ferguson *et al.* (2014)
Lotus japonicus								
LjCLE-RS1	93	LjHAR1	Systemic	Rhizobia and nitrate	Early (<24 h)	Y (100%)	Y	Okamoto *et al.* (2009)
LjCLE-RS2	81	LjHAR1	Systemic	Rhizobia	Early (<24 h)	Y (100%)	Y	Okamoto *et al.* (2009)
Medicago truncatula								
MtCLE12	81	MtSUNN	Systemic	Rhizobia	Late (~4–6 days)	N (<40%)	N	Mortier *et al.* (2010)
MtCLE13	84	MtSUNN	Systemic	Rhizobia	Early (<4 days)	Y (100%)	Y	Mortier *et al.* (2010)

Table 2. Amino acid sequence similarity (%) amongst the known nodulation CLE prepropeptides
Similarities are based on alignments obtained using CLUSTALW multiple alignment tool in Geneious Pro 6.0

	GmRIC1	GmRIC2	GmNIC1	PvRIC1	PvRIC2	PvNIC1	LjCLE-RS1	LjCLE-RS2	MtCLE12
GmRIC2	49.9	–	–	–	–	–	–	–	–
GmNIC1	26.4	24.1	–	–	–	–	–	–	–
PvRIC1	68.7	61.1	15.4	–	–	–	–	–	–
PvRIC2	47.4	82.1	21.8	40.4	–	–	–	–	–
PvNIC1	21.4	30.7	69.1	22.0	23.0	–	–	–	–
LjCLE-RS1	42.3	47.9	26.2	40.4	48.4	24.4	–	–	–
LjCLE-RS2	45.8	41.5	24.4	43.0	34.0	27.2	34.4	–	–
MtCLE12	32.6	33.0	22.6	30.9	32.6	25.0	40.2	28.4	–
MtCLE13	37.9	44.1	25.0	37.8	43.0	26.3	51.6	42.0	30.6

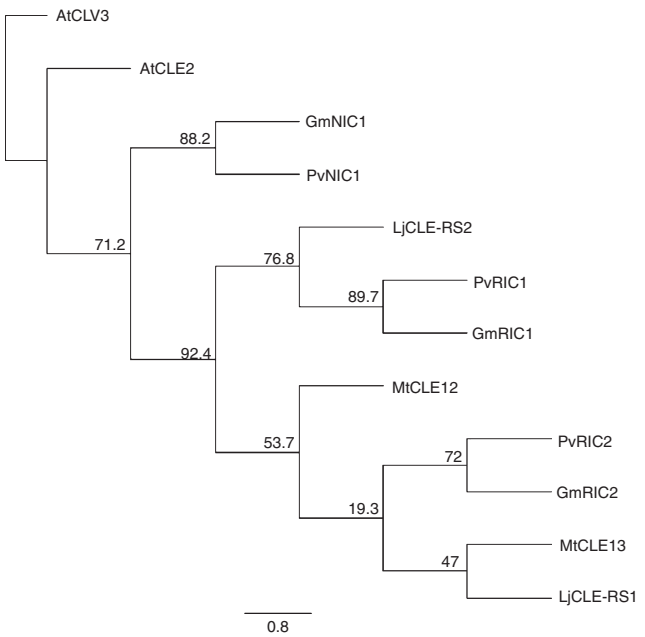

Fig. 2. Phylogenetic tree of nodulation CLE prepropeptides. The known nodulation-suppressing CLE peptides of *Medicago truncatula*, *Lotus japonicus*, *Glycine max* (soybean) and *Phaseolus vulgaris* (common bean) are shown, together with AtCLV2, the *Arabidopsis* CLE peptide most similar to the nodulation CLE peptides, and AtCLV3 as an outgroup. The tree was generated using PhyML 3.0 (Guindon and Gascuel 2003) in Geneious Pro 6.0 and constructed using the maximum likelihood approach. A branch was supported in 1000 bootstrap replications, with bootstrap confidence values expressed as a percentage of the 1000 bootstrap replications (Felsenstein 1985).

similar role for this domain is predicted for the nodulation CLE peptides.

Amongst the known nodulation CLE prepropeptides, the signal peptide domain has a 37% pairwise identity and contains a leucine-rich motif (Fig. 1), commonly observed in exported proteins (la Cour *et al.* 2004). Also present within this domain is a conserved motif of five amino acids (TLQAR; Table 1), which is predicted to be a site of cleavage (Okamoto *et al.* 2009). It has been noted that GmNIC1, PvNIC1 and MtCLE12 show lower conservation of amino acid residues within this motif. They are also the only known nodulation CLE peptides to lack the *C*-terminal extension domain (Table 1; Fig. 1). Outside of this motif, conserved sequence residues within the signal peptide can be seen amongst predicted orthologues of the nodulation CLE peptides (Fig. 1; Reid *et al.* 2011*a*; Ferguson *et al.* 2014).

Variable region

The functional importance of the variable domain, the least conserved of the four domains, remains unknown (Ni and Clark 2006; Meng *et al.* 2010; Reid *et al.* 2013). Indeed, AtCLV3 shows function without this domain (Fiers *et al.* 2006). The size of the domain is also highly variable (31–50 amino acids). However, recognition and cleavage immediately before the Arg1 residue of the CLE domain requires at least four to five residues of the variable domain to be present for correct processing of AtCLV3 (Kondo *et al.* 2008; Ni *et al.* 2011; Xu *et al.* 2013). An additional amino acid at residue 39 within the variable domain of AtCLV3 is also predicted to be a cleavage site (Xu *et al.* 2013).

There are no residues that are 100% conserved across the variable domain between the known nodulation CLE peptides (Fig. 1), although it shows a 19.4% pairwise identity and, as with other domains, residues are conserved between orthologues. This is particularly evident between the nodulation CLE peptides of the closely-related bean and soybean species (Fig. 1; Ferguson *et al.* 2014).

CLE domain

The CLE domain, from which the peptide is named, denotes the mature/active peptide sequence. It is located at the *C*-terminus and is the most conserved region (Cock and McCormick 2001; Oelkers *et al.* 2008). The consensus amino acid sequence of the nodulation-suppressing CLE peptides is RL(A/S)PGGPDPQHN(X) (Fig. 1). The domain is 12 or 13 amino acids in length and contains 50% identical sites, with 77.4% (12 amino acids) and 75.2% (13 amino acids) pairwise identity between the known nodulation CLE peptides. LjCLE-RS2 of *L. japonicus* is the only structurally-confirmed nodulation CLE peptide, and is 13 amino acids in length (Okamoto *et al.* 2013). However, the nitrate-induced GmNIC1 peptide of soybean and its orthologue in bean, PvNIC1, have a stop codon at position 13 and therefore can only be 12 amino acids in length (Fig. 1; Reid *et al.* 2011*a*; Ferguson *et al.* 2014). This may influence their

functional properties, such as their apparent lack of long distance transport.

Notably, GmRIC1, PvRIC1, GmRIC2, and PvRIC2 are the only nodulation CLE peptides known to contain an Ala residue at position 3 of the CLE domain, presumably a result of polyploidisation and subsequent species divergence amongst the legumes (Figs 1, 2; Stefanović *et al.* 2009; Schmutz *et al.* 2010). There are four other residues within the CLE domain of the known nodulation CLE peptides that contain sequence divergence from the consensus sequence: Gly5 > Glu5 (GmRIC1 and PvRIC1) or Ala5 (MtCLE13); Asp8 > Asn8 (MtCLE12); Pro9 > His9 (MtCLE12) or Gln9 (GmNIC1); and Gln10 > His10 (GmRIC1 and PvRIC1) or Ile10 (MtCLE12) (Fig. 1). It is not yet known how the activity of the CLE peptide is affected by these sequence divergences. Only the one at position 8 in MtCLE12 is predicted to be critical for function (i.e. the suppression of nodulation) based on site-directed mutagenesis work using soybean (Reid *et al.* 2013); however, this nonsynonymous substitution from an uncharged asparagine to a negatively charged aspartic acid is conservative and may not affect activity.

Recent research has indicated that, despite sequence-redundancy of the CLE domain, there is likely some specificity between pathways and/or species that are dependent on sequence. Okamoto *et al*. (2013) were unable to elicit a plant response in *L. japonicus* from exogenous application of the mature AtCLV3 peptide, but saw a reduction in nodules when LjCLE-RS2 was applied with the correct post-translational modifications. Chimeric genes that swapped the CLE domains of GmNIC1 and GmRIC1 also impacted on the suppression of nodulation compared with their respective native genes (Reid *et al*. 2013). In contrast, *GmRIC1* overexpression in common bean and *MtCLE13* overexpression in pea strongly suppressed nodulation inter-specifically (Osipova *et al.* 2012; Ferguson *et al.* 2014), indicating that these CLE peptide encoding genes can function in the AON pathways of other legume species. However, overexpression results of any kind should always be interpreted with care.

C-terminal extension domain

The *C*-terminal domain of the known nodulation-suppressing CLE peptides is small, at ~6–9 residues in length, and is even completely absent from some (Fig. 1; Table 1). Indeed, of the known nodulation CLE peptides, GmNIC1, PvNIC1 and MtCLE12 all lack the *C*-terminal extension in its entirety (Mortier *et al.* 2010; Reid *et al.* 2011*a*; Ferguson *et al.* 2014). However, the remaining nodulation CLE peptides all contain the domain, as do AtCLV3 and GmCLV3 of the CLAVATA pathway (Fiers *et al.* 2006; Wong *et al.* 2013).

The *C*-terminal domain is thought to act as a protective mechanism from degradative protease enzymes in the xylem, which the peptides would encounter during systemic transport (Oelkers *et al.* 2008; Okamoto *et al.* 2009; Ni *et al*. 2011; Reid *et al.* 2011*a*). It is characteristic of the rhizobia-dependent, systemically acting, CLE peptides, and is not present in the nitrate-induced, locally-acting GmNIC1 of soybean and its orthologue in bean, PvNIC1 (Reid *et al.* 2011*a*; Ferguson *et al.* 2014). This would appear to further support a role for the domain in protection during long-distance xylem transport. Moreover, overexpressing a chimeric construct that added the *C*-terminal domain of GmRIC1 to GmNIC1 enhanced the suppression of nodulation compared with that of the native GmNIC1 (Reid *et al.* 2013). In contrast, the removal of the domain from GmRIC1 did not alter its ability to suppress when overexpressed (Reid *et al.* 2013), but this may be due to the overexpression technique masking or over-compensating for the true function of the modified construct. MtCLE12 also lacks the *C*-terminal domain and is both induced by rhizobia and predicted to be transported systemically (Mortier *et al.* 2010), so the exact need for the domain remains puzzling.

Two conserved proline residues are present within the *C*-terminal extension of all seven nodulation CLE peptides that contain the domain (Fig. 1; Okamoto *et al.* 2009; Mortier *et al.* 2010; Reid *et al.* 2011*a*; Ferguson *et al.* 2014). Site-directed mutagenesis and overexpression of *GmRIC1* modified to encode two alanine residues in place of these two proline residues did not alter the suppressive activity of the peptide, consistent with the unclear role of this domain (Reid *et al.* 2013).

Post-translational modifications and critical residues of the CLE domain

The mature nodulation-suppressive CLE peptide of *L. japonicus*, LjCLE-RS2, is 13 amino acids in length and is hydroxylated at Pro4 and Pro7, with Hyp7 further modified to contain three arabinose sugars connected via β-1-2-linkages. These modifications are predicted to be made in the extracellular fluids (Okamoto *et al.* 2013). This is consistent with mature AtCLV3, AtCLE2 and AtCLE9 glycopeptides, which also contain a Hyp7 having three linked L-arabinose sugars (Kondo *et al.* 2006; Ohyama *et al.* 2009; Okamoto *et al.* 2013; Shinohara and Matsubayashi 2013). All of the nodulation CLE peptides contain motifs associated with arabinose modifications that are present in other plant proteins/peptides (Matsubayashi 2014). The hydroxyproline O-arabinosyltransferase (HPAT) gene that controls CLE arabinosylation in *Arabidopsis* is called *AtHPAT3* (Ogawa-Ohnishi *et al.* 2013). *MtRDN1* and *PsNOD3* are likely orthologues of *AtHPAT3* and are thought to be responsible for the arabinosylation of the nodulation-suppressing CLE peptides (Ogawa-Ohnishi *et al.* 2013). Mutations in these genes result in a supernodulation phenotype (Jacobsen and Feenstra 1984; Postma *et al*. 1988; Sagan and Duc 1996; Li *et al.* 2009; Schnabel *et al.* 2011), indicating that the peptides require the arabinose sugars for their activity.

Application of synthesised arabinosylated-LjCLE-RS2 to leaves of *L. japonicus* plants caused a reduction in nodulation in an *LjHAR1*-dependent manner (Okamoto *et al.* 2013). However, root or shoot application of synthetic nodulation CLE peptides devoid of modifications did not affect nodulation, although altered root growth was observed (Okamoto *et al.* 2009; Saur *et al.* 2011). Moreover, application of AtCLV3 with the arabinose modifications also had no effect on nodulation (Okamoto *et al.* 2013). Shinohara and Matsubayashi (2013) demonstrated that the binding of the AtCLV3 CLE peptide to the AtCLV1 LRR receptor-kinase declined as the arabinose chain length decreased, whereas AtCLE9 showed no change in receptor binding efficacy to its

receptor, BAM1, a CLV1/BAM-family LRR RK, in the absence of the arabinose chain (Shinohara *et al*. 2012). Further, tracheary element differentiation inhibitory factor (TDIF) peptides synthesised with or without hydroxyproline residues can mimic the function of the naturally occurring peptide, which contains Hyp4 and Hyp7 (Sawa *et al.* 2006).

In addition to post-translational modifications to critical residues, the structural configuration of the CLE peptide ligand is also likely to impact markedly on receptor interactions. Gly6 is proposed to allow for rotation, most likely because of its small size, complementing evidence for a boomerang curve in the peptide's configuration, with both ends of the peptide bending away from the arabinosylation at Hyp7 (Okamoto *et al.* 2013; Shinohara and Matsubayashi 2013; Song *et al.* 2013). Notably, Gly6 is 100% conserved amongst the known nodulation CLE peptides (Fig. 1). Site-directed mutagenesis of Gly6 to Ala6 significantly reduced the nodule suppressive activity of GmRIC1 (Reid *et al.* 2013). Song *et al*. (2013) altered Gly6 of AtCLV3 into 18 other amino acids; no substitution was able to rescue the phenotype of *Atclv3* mutant plants. Similar specificity is expected for the nodulation CLE peptides. In addition to Gly6 and Pro7, residues Arg1, Pro4, Asp8, His11 and Asn12 of GmRIC1 were required for full nodulation-suppression activity in soybean (Reid *et al.* 2013). Similarly, TDIF also lost activity when the CLE domain residues His1, Val3, Gly6, Asn8, Pro9 and Asn12 were changed into an alanine residue via site-directed mutagenesis (Ito *et al.* 2006; Sawa *et al.* 2006).

It has been noted that locally-acting CLE peptides, including GmNIC1 and PvNIC1 (Fig. 1), in addition to AtCLV3, GmCLV3 and LjCLV3, all contain His12 (Reid *et al.* 2011*a*; Okamoto *et al.* 2011; Wong *et al.* 2013; Ferguson *et al.* 2014). This may indicate a role for this residue in local, but not systemic, transport of the peptide. Constructs having swapped the CLE domain of the systemically-acting GmRIC1 and the locally-acting GmNIC1 showed an altered inhibition of nodulation when overexpressed compared with the native peptides (Reid *et al.* 2013). Whether residue 12 plays a specific role in the transport or recognition of the peptide is of interest to determine.

As noted above, Arg1 of the AtCLV3 CLE domain has been shown to be critical for binding and processing of the mature CLE peptide, with at least 4–5 residues upstream of Arg1 required for proper recognition of the signal (Kondo *et al.* 2008; Ni *et al.* 2011; Xu *et al.* 2013). It is hypothesised that a subtilisin with endoproteolytic activity cleaves the CLE peptide, with a carboxypeptidase processing the C-terminal extension where present (Ni *et al.* 2011; Djordjevic *et al*. 2011). However, to date, there is little known about the mechanisms and sites of proteolytic cleavage in the nodulation CLE peptides.

Mode of induction of the nodulation-suppressing CLE peptides

All of the known nodulation-suppressing CLE peptides are upregulated in expression by the presence of rhizobia and/or the available soil nitrogen content (Table 1). Phylogenetic analysis shows that they cluster according to their mode of induction (Fig. 2). Evidence for other environmental factors such as phosphate and soil acidity, inducing or influencing the expression of CLE peptide-encoding genes also exists.

Rhizobia-induced CLE peptides

The presence of compatible rhizobia, and possibly more specifically the rhizobia-produced Nod factor signal, elicits the expression of systemically-acting CLE peptide-encoding genes that function in AON. These CLE peptides include: *LjCLE-RS1*, *LjCLE-RS2*, *MtCLE12*, *MtCLE13*, *GmRIC1*, *GmRIC2*, *PvRIC1*, and *PvRIC2* (Table 1; Okamoto *et al.* 2009, 2013; Mortier *et al.* 2010, 2012; Lim *et al.* 2011; Reid *et al.* 2011*a*, 2013; Saur *et al.* 2011; Hayashi *et al.* 2012; Ferguson *et al.* 2014). Overexpression of these peptides in wild-type legume plants results in a complete abolishment of nodulation, but does not alter the nodulation pattern in NARK mutants, demonstrating that they act in a NARK-dependent manner (Okamoto *et al.* 2009; Mortier *et al.* 2010; Reid *et al.* 2011*a*; Lim *et al.* 2011).

Laser microdissection of root sections indicate that *LjCLE-RS1* and *-RS2* are expressed in the stele and outside of the endodermis (cortex and epidermis) (Okamoto *et al.* 2009). Promoter:*GUS* reporter fusion studies have shown that *MtCLE13* is expressed in the inner cortex during early nodulation and later in dividing cells of the cortex and pericycle. In contrast, MtCLE12 is not expressed early but instead is expressed throughout young nodules and in meristematic tissues of the elongating indeterminate nodule (Mortier *et al.* 2010). Finally, Lim *et al*. (2011) have shown that *GmRIC2* is expressed in the pericycle and inner cortex during early nodule development, and later in the outer cortex of more developed nodules.

Time-course experiments have revealed different but overlapping expression patterns for these genes within a species (Table 1). Soybean *GmRIC1* is induced early (within 12 h) after inoculation with infection-capable (Nod factor producing) *Bradyrhizobium japonicum*, whereas *GmRIC2* expression is induced later (48–72 h) and remains elevated in expression for longer (Reid *et al.* 2011*a*; Hayashi *et al.* 2012). The rhizobia-induced peptide encoding genes of common bean, *PvRIC1* and *PvRIC2*, exhibit a similar pattern of expression (Ferguson *et al.* 2014). Likewise, *M. truncatula MtCLE13* is expressed earlier than *MtCLE12*, although both are also expressed in later stages of nodulation (Mortier *et al.* 2010, 2012). *LjCLE-RS1* and *LjCLE–RS2* are both upregulated within 3 h of inoculation (Okamoto *et al.* 2009). Similar to *GmRIC2*, *MtCLE13* and *LjCLE-RS1* transcript levels appear to remain elevated for longer compared with *MtCLE12* and *LjCLE–RS2*, respectively (Okamoto *et al.* 2009; Mortier *et al.* 2010; Reid *et al.* 2011*a*).

When compared with wild-type plants, a significant increase in expression of both *LjCLE-RS1* and *LjCLE–RS2* was also observed in the hypernodulating mutant of *L. japonicus*, *too much love*, possibly indicating that their synthesis is directly linked to the number of nodules being formed (Magori and Kawaguchi 2010). Interestingly, the plant hormone cytokinin, which has a role in early nodule development (reviewed in Ferguson and Mathesius 2014), has also been shown to induce the expression of some nodulation-suppressing CLE peptide genes (Lim *et al*. 2011; Mortier *et al*. 2010, 2012), consistent with the idea that the initiation of the AON pathway is linked to early cell divisions. Additional studies are required to further understand the expression patterns of these nodulation-suppressing CLE peptides, both within and between species.

Nitrate-induced CLE peptides

GmNIC1 and *LjCLE-RS2* are the only nodulation-suppressing CLE peptide-encoding genes that are confirmed to respond to nitrate (Table 1; Okamoto *et al.* 2009; Reid *et al.* 2011*a*). *GmNIC1* is specifically induced by nitrate and not co-induced by the rhizobial microsymbiont, whereas *LjCLE-RS2* is reported to be induced by both. *PvNIC1* is also likely to be induced by nitrate as the candidate orthologue of *GmNIC1* (Ferguson *et al.* 2014). To date, no CLE peptide-encoding gene of *M. truncatula* has been reported to respond to nitrate, although evidence suggests the existence of a locally acting, nitrate-responsive mechanism that acts in a MtSUNN-dependent manner to regulate nodulation (Jeudy *et al.* 2010). We note that *AtCLE2*, the *Arabidopsis* gene most similar to the nodulation-suppressing CLE peptides, has a role in root development and is also induced by nitrate (Scheible *et al.* 2004; Araya *et al.* 2014).

Overexpression of the locally-acting *GmNIC1* in wild-type soybean reduces nodule numbers by ~50% compared with empty vector controls (Reid *et al.* 2011*a*). Although significant, this suppressive ability is far from that of *GmRIC1* and *GmRIC2*, as discussed above. Confirmation is required to determine whether this is unique to soybean or is shared with the closely related orthologues identified in common bean (Ferguson *et al.* 2014).

Other inducing factors

Numerous factors can influence the extent of nodulation and it is possible that some do so by inducing, or otherwise influencing, the production, transport, perception or response to a CLE peptide(s). Recently, split-root and grafting studies using soybean grown in low pH conditions revealed a novel systemic mechanism that acts via GmNARK in the shoot to inhibit nodulation of the root (Lin *et al.* 2012; Ferguson *et al.* 2013). This suggests that soil acidity may act via a CLE peptide to suppress nodulation. Two CLE peptide-encoding genes of *L. japonicus*, called *LjCLE19* and *LjCLE20*, have been shown to be upregulated in the presence of phosphate (Funayama-Noguchi *et al.* 2011); however, a specific role for these peptides in plant development has not been reported. It has been noted that although CLE peptides are nearly-exclusive to plants, they also exist in plant-parasitic nematodes (e.g. Bakhetia *et al.* 2007), which appear to use the peptides to initiate the formation of feeding structures in host roots (reviewed by Mitchum *et al.* 2012). Also noted is that nematodes are easily genetically transformed through simple feeding, suggesting that perhaps nematode CLE genes were plant-derived. Whether nematodes, or any other pathogen, can also induce a plant-encoded CLE peptide(s) is of great interest to determine.

Rhizobia-induced CLE peptides that do not suppress nodulation

In addition to the nodulation-suppressing CLE peptides reported above, two further CLE peptide-encoding genes have been identified that are expressed in response to rhizobia inoculation, namely *LjCLE3* (Okamoto *et al.* 2009) and *MtCLE4* (Mortier *et al.* 2010). Overexpression of these genes does not alter the nodulation phenotype when compared with empty vector controls. It is now of interest to determine the role of these peptides in nodulation to determine why they are responsive to rhizobia inoculation and how they function in this symbiosis.

Future perspectives

The role of CLE peptides functioning as hormone signals in plant development is only just beginning to emerge, with the activity of most remaining to be elucidated. How and where the CLE peptides are induced, whether they are transported and act locally or systemically, how they are perceived, the downstream signals they induce, and their precise role in various plant developmental pathways are all features of great interest to establish in this burgeoning research field. Indeed, establishing the function of CLE peptides acting in critical plant developmental processes will considerably help to advance the current molecular knowledgebase of a variety of plant signalling networks.

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