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BOTANICAL BRIEFING

Molecular mechanisms controlling legume autoregulation of nodulation

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• *Background* High input costs and environmental pressures to reduce nitrogen use in agriculture have increased the competitive advantage of legume crops. The symbiotic relationship that legumes form with nitrogen-fixing soil bacteria in root nodules is central to this advantage.
• *Scope* Understanding how legume plants maintain control of nodulation to balance the nitrogen gains with their energy needs and developmental costs will assist in increasing their productivity and relative advantage. For this reason, the regulation of nodulation has been extensively studied since the first mutants exhibiting increased nodulation were isolated almost three decades ago.
• *Conclusions* Nodulation is regulated primarily via a systemic mechanism known as the autoregulation of nodulation (AON), which is controlled by a CLAVATA1-like receptor kinase. Multiple components sharing homology with the CLAVATA signalling pathway that maintains control of the shoot apical meristem in arabidopsis have now been identified in AON. This includes the recent identification of several CLE peptides capable of activating nodule inhibition responses, a low molecular weight shoot signal and a role for CLAVATA2 in AON. Efforts are now being focused on directly identifying the interactions of these components and to identify the form that long-distance transport molecules take.

Key words: Legume nodulation, AON, signalling, hormone, plant peptide, receptor kinase, symbiosis.

INTRODUCTION

Nitrogen is a component of many biological molecules, making its availability critical to sustained plant growth and reproduction. Atmospheric nitrogen gas is plentiful but is unavailable to most organisms. Many legumes overcome this limitation by initiating a symbiotic relationship with soil bacteria, collectively referred to as rhizobia. These rhizobia are capable of biological nitrogen fixation where atmospheric nitrogen (N_2) is fixed by the nitrogenase enzyme complex of the endocytotic bacteria when they reside inside legume organs called nodules. Nodules provide the rhizobia with an energy source from photoassimilates (as malate; Udvardi *et al.*, 1988) while maintaining the low oxygen environment required for efficient nitrogen fixation. The process of nodule formation and subsequent nitrogen fixation is balanced with the plant's own energy requirements in a process termed autoregulation of nodulation (AON; see Caetano-Anollés and Gresshoff, 1991; Ferguson *et al.*, 2010). Nodulation is also regulated in response to nitrogen availability in the soil (Carroll *et al.*, 1985; Ferguson and Mathesius, 2003).

This review highlights significant progress that has recently been made in the identification of new genes and factors controlling nodulation. Further understanding of the mechanisms that allow plants to balance nitrogen resources with their energy demands may enable nitrogen use optimization in important legume crops, including the most widely grown soybean. Significant agricultural, economic and environmental benefits stand to be gained by further reducing nitrogen fertilizer inputs while maintaining or improving legume yields.

AUTOREGULATION OF NODULATION

Nodulation occurs in a distinct pattern where nodules form in the region having susceptible root hairs at the time of inoculation [zone of nodulation (ZON); Bhuvaneswari *et al.*, 1981; Calvert *et al.*, 1984]. AON causes a nodulation phenotype where the majority of nodules form near the crown of the root system. It is unclear which stage of nodule development AON inhibits, although approach grafting in *Pisum sativum* indicated that the onset of AON is triggered before extensive cell divisions are observed (Li *et al.*, 2009). This supports anatomical observations in soybean where a significant reduction of nodule development stages occurs along the root (Mathews *et al.*, 1989) and split-root experiments where inoculation is delayed by 3–4 d (Kosslak and Bohlool, 1984; Olsson *et al.*, 1989). It has been proposed that AON reduces the speed of cortical cell divisions to restrict nodulation at the early stages when these cell divisions are occurring (Mathews *et al.*, 1989).

The precise nature and co-ordination of these regulatory cues remain to be elucidated; however, the maintenance of autoregulation in spontaneous nodulation mutants indicates that a bacterial-derived signal is not the elicitor of AON and that it is not entirely dependent on nitrogen fixation in the nodule (Caetano-Anollés *et al.*, 1990; Tirichine *et al.*, 2006, 2007). The precise onset of AON is also not known, although experimental evidence suggests that AON is effective as early as 4 d after inoculation (Kosslak and Bohlool, 1984; Olsson *et al.*, 1989; Suzuki *et al.*, 2008).

Studies using grafting and split-root techniques have shown that AON is induced systemically by a graft-transmissible

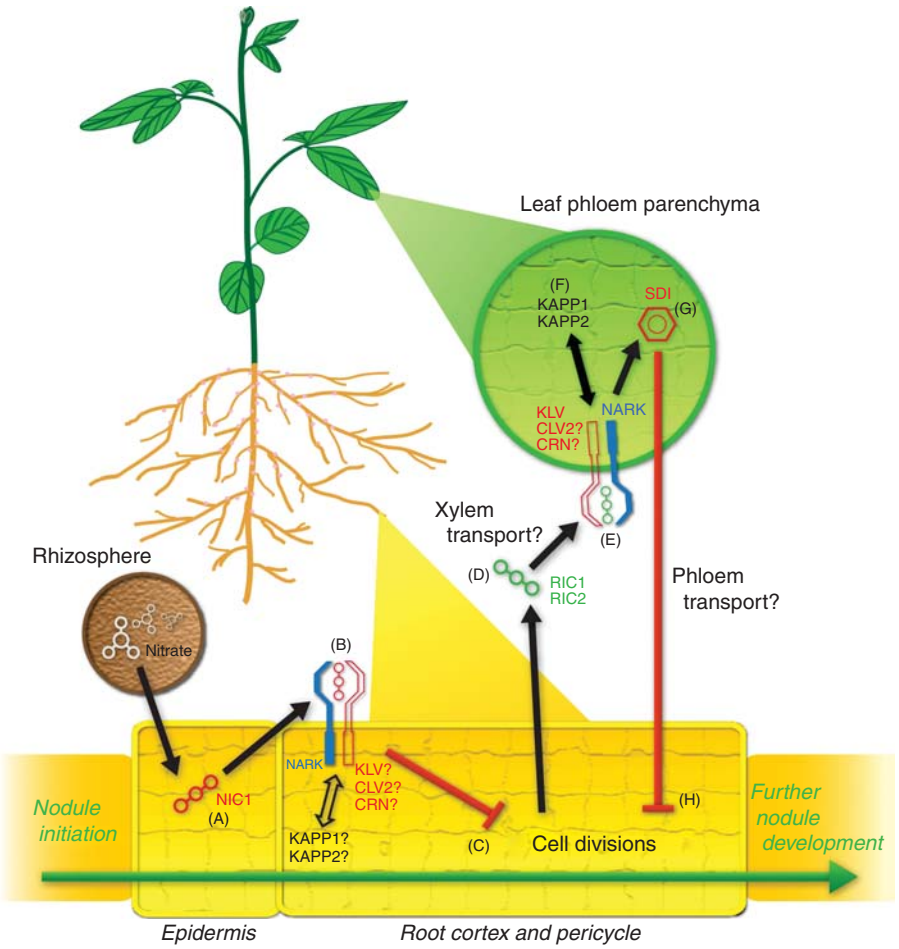

FIG. 1. A working model of root and shoot mechanisms in autoregulation of nodulation (AON). Legumes regulate nodulation in response to pre-existing infections and soil nitrogen levels. Nitrate induces the production of a nitrate-induced CLE peptide (NIC1; A) that acts locally in the root via the AON receptor kinase, NARK (B; or its orthologues in other species), to inhibit nodule progression (C). NARK may act in concert with other components to perceive NIC1. Rhizobia-induced CLE peptides (RICs) are induced at several stages of nodule development and may be transported via the xylem (D) to the shoot. In the shoot, NARK and possibly also CLV2, KLV and CRN are required for the perception of these putative ligands (E). Two kinase-associated protein phosphatases (KAPP1/2) are phosphorylated by NARK and in turn dephosphorylate the NARK kinase (F). An equilibrium of phosphorylation between these components may be required preceding the production of the shoot-derived inhibitor (SDI; G). SDI is transported via the phloem to the roots where it inhibits further nodule progression and cell divisions (H). A compound similar to SDI may also be involved in the nitrate pathway that acts locally to inhibit the progression of nodule formation (C).

signal and is controlled by the shoot (Delves *et al.*, 1986, 1992). These experiments led to a model where a root-derived nodulation signal is the cue (Q) for the onset of AON. Subsequently, a shoot-derived inhibitor (SDI) is transported back down to the roots where it regulates further nodulation events (Fig. 1D–H).

In addition to the systemic AON mechanism where plants regulate nodulation in response to existing infection events, legumes also regulate nodulation in response to environmental nitrogen availability. This may represent a means of preferentially obtaining nitrogen from sources that are energetically favourable relative to the energy costs of nodulation. Nodulation tolerant to high soil nitrate levels has been used as a screening method to identify nodule regulation mutants (e.g. *nod3*, Jacobsen and Feenstra, 1984; *nts*, Carroll *et al.*, 1985). The *nts* mutants of soybean exhibit a supernodulation phenotype in both high and low nitrate conditions, indicating that the AON and nitrate regulation pathways share genetic components (Day *et al.*, 1986). Evidence exists for both local and systemic regulatory mechanisms functioning in response to nitrate, and it is likely that multiple mechanisms are acting in concert (Hinson, 1975; Cho and Harper, 1991; Okamoto *et al.*, 2009; Jeudy *et al.*, 2010; Reid *et al.*, 2011).

ROOT-DEPENDENT COMPONENTS OF AON

Autoregulation of nodulation can be divided into root- and shoot-dependent components (Fig. 1). The former components are likely to include factors involved in the response to initial cell divisions that lead to the induction of Q. A second class of root-dependent components are those which act downstream of shoot signalling to perceive the SDI signal and/or that inhibit further nodule progression.

TABLE 1. *Genes/mutants and products involved in legume regulation of nodule number*

Gene/mutant	Gene product	Site of production	Site of action	Comments	References
GmNARK; *GsNARK*; *LjHAR1*; *MtSUNN*; *PsSYM29*	LRR-RK	Shoot/root	Shoot/root	Acts in shoot (AON) and root (NO_3^- inhibition) (LRR-RK)	Sagan and Duc (1996); Krusell *et al.* (2002); Men *et al.* (2002); Nishimura *et al.* (2002*a*); Searle *et al.* (2003); Schnabel *et al.* (2005)
GmNIC1	CLE	Root	Root	NO_3^- induced (CLE pre-propeptide)	Reid *et al.* (2011)
GmRIC1/2; *LjCLE-RS1/2*; *MtCLE12/13*	CLE	Root	Probably the shoot	Rhizobia-induced (CLE prepropeptide)	Okamoto *et al.* (2009); Mortier *et al.* (2010); Reid *et al.* (2011); Lim *et al.* (2011)
LjASTRAY	bZIP TF		Root	Also acts in photomorphogenesis (transcription factor)	Nishimura *et al.* (2002*b*)
LjCLV2; *PsSYM28*	CLV2	Shoot/root	Shoot/root?	May interact with other AON LRR RKs (truncated LRR-receptor protein)	Sagan and Duc (1996); Krusell *et al.* (2011)
LjETR1	ETR1	Shoot/root	Shoot/root	Ethylene receptor (two-component receptor)	Gresshoff *et al.* (2009); Lohar *et al.* (2009)
LjKLV	LRR-RK	Shoot/root?	Shoot/root?	May interact with other AON LRR RKs	Oka-Kira *et al.* (2005)
LjPLENTY	Unknown	Root	Root	Hypernodulation phenotype	Yoshida *et al.* (2010)
LjRDH1	Unknown	Root	Root		Ishikawa *et al.* (2008)
LjTML	Unknown	Root	Root		Magori *et al.* (2009)
MtEFD	AP2-EREBP TF	Root	Root	Positively regulates CK levels (transcription factor)	Vernié *et al.* (2008)
MtLSS	Unknown	Shoot/root?	Shoot/root?	Possible epigenetic factor of *MtSUNN*	Schnabel *et al.* (2010)
MtSKL	EIN2	Root	Root	\ethylene response factor	Penmetsa and Cook (1997); Penmetsa *et al.* (2008)
PsNOD1 and *2*	Unknown				Gelin and Blixt (1964)
PsNOD3; *MtRDN1*	RDN1	Root	Root	Affects CLE synthesis and/or transport	Jacobsen and Feenstra (1984); Engvild (1987); Novák *et al.* (1997); Li *et al.* (2009); Schnabel *et al.* (2011)
PsNOD4 and *5*	Unknown		Shoot		Sidorova and Shumnyi (1998, 2003)
PsNOD6	Unknown		Shoot		Sidorova and Shumnyi (1998)

Several mutants have been isolated that exhibit an increased nodulation phenotype, known as hyper- or supernodulation (see Table 1). Those functioning in the root include *rdh1* (Ishikawa *et al.*, 2008), *rdn1* (Schnabel *et al.*, 2011), *nod3* (Jacobsen and Feenstra, 1984), *too much love* (Magori *et al.*, 2009), *plenty* (Yoshida *et al.*, 2010), *efd-1* (Vernié *et al.*, 2008), *astray* (Nishimura *et al.*, 2002*b*) and *sickle* (Penmetsa *et al.*, 2008). Each of these mutants forms an overabundance of nodules, though some differences are noted in their pattern and extent of nodulation. In addition, not all of these factors function directly in the AON pathway.

Approach grafting indicated that *PsNOD3*, the homologue of *MtRDN1*, may function prior to the shoot responses, possibly in the production or transmission of the root-derived signal (Li *et al.*, 2009). In contrast, *TOO MUCH LOVE* inhibits nodulation locally and may act downstream of the shoot components, possibly as a receptor for the SDI signal or in a related function (Magori *et al.*, 2009).

CLE PEPTIDES IN AON

The first genes to be identified in AON were those encoding a group of orthologous leucine-rich repeat (LRR) receptor kinases similar to CLAVATA1 (CLV1; Clark *et al.*, 1997) in arabidopsis (*LjHAR1*, Krusell *et al.*, 2002; Nishimura *et al.*, 2002*a*; *PsSYM29*, Krusell *et al.*, 2002; *GmNARK*, Searle *et al.*, 2003; Men *et al.*, 2002; and *MtSUNN*, Schnabel *et al.*, 2005). Mutations in these genes reduce the plant's ability to regulate nodule numbers and, in all cases tested, nodulation is tolerant to otherwise inhibitory high nitrate conditions.

The similarity of these AON receptor kinases to CLV1 prompted searches for ligands related to the CLV3 peptide, the ligand of CLV1 (Fletcher *et al.*, 1999; Kondo *et al.*, 2006, 2008; Oelkers *et al.*, 2008; Ogawa *et al.*, 2008). CLE peptides responding to inoculation were identified which can systemically reduce nodule numbers in *Lotus japonicus* (Okamoto *et al.*, 2009). Related peptides with AON receptor-dependent activity have since been identified in *Medicago truncatula* (Mortier *et al.*, 2010; Saur *et al.*, 2011) and *Glycine max* (Reid *et al.*, 2011; Mortier *et al.*, 2011; Lim *et al.*, 2011). The expression of these CLE peptide-encoding genes suggests they are induced in response to inoculation and the onset of nodule development. However, the peptides themselves have yet to be directly detected *in planta* and they have not been confirmed to move long distances as is expected if they are perceived in the shoot. Some of these CLE peptide-encoding genes (*LjCLE-RS2* and *GmNIC1*) were also found to be responsive to nitrate, highlighting the mechanistic and functional similarities between AON and the nitrate regulation of nodulation. GmNIC1 differed from the other CLE peptides so far identified as it functions locally via NARK to regulate nodulation (Fig. 1A–C) and does not appear to be induced by the rhizobia.

There appears to be some functional divergence between the inoculation-responsive CLE peptides as the timing of their induction was found to be variable. The expression of *LjCLE-RS1/2*, *MtCLE13* and *GmRIC1* is induced early in

response to inoculation, whereas *GmRIC2*, *MtCLE12* and *MtCLE13* were persistent later when mature nodules were present. Li and associates (2009) showed that AON activation requires signalling at several nodule developmental stages, indicating that multiple signals may be required for activation and maintenance.

The secondary structure of CLE pre-propeptides can be characterized by the presence of a 5′ signal peptide which is likely to be required for the cellular export and localization properties of the peptide (Meng *et al.*, 2010). CLE peptides also possess a 12–13 amino acid motif deemed to represent the final active peptide which is cleaved from close to the 3′ end of the initial protein (Oelkers *et al.*, 2008). Outside of the signal peptide and the CLE motif there appears to be little sequence conservation within CLE proteins. However, the CLE peptides capable of regulating nodulation share some common features outside of these more general CLE characteristics (Okamoto *et al.*, 2009; Mortier *et al.*, 2010; Reid *et al.*, 2011). Within the signal peptide region, a well-conserved motif was identified, although apart from the predicted export role the exact function of this remains obscure. Several of these CLE peptides also possess a 5–7 amino acid conserved extension beyond the 3′ CLE motif. CLE peptides require processing to generate the final product and several protease cleavage events may be required (Ni and Clark, 2006; Ni *et al.*, 2011). The extracellular fluid of legumes has been demonstrated to possess factors with proteolytic activity capable of producing biologically active CLE peptides (Djordjevic *et al.*, 2011). The activity of CLE peptides has also been shown to be dependent on post-translational modifications, including hydroxylation of proline residues and glycosylation of key residues (Kondo *et al.*, 2006; Ohyama *et al.*, 2009). In arabidopsis, CLV3 and CLE2 (which share a similar CLE domain sequence to the nodulation CLE peptides) were identified with three β-1,2-linked arabinose moieties bound to Hyp7 of the 13 amino acid CLE peptide (Ohyama *et al.*, 2009).

Due to the systemic signalling requirement in AON, it is presumed that long-distance transport of the Q signal is required, probably via the xylem (Fig. 1D). Efforts have therefore been undertaken to characterize the protein and metabolite components of the xylem sap of legume plants. The soybean xylem sap proteome identified several protein components, although none of these differed between plants with or without nodules (Djordjevic *et al.*, 2007). There were, however, changes observed in the xylem sap proteome of inoculated soybeans at the seedling stage, though a distinct role for these changes in nodulation was not identified (Subramanian *et al.*, 2009).

SHOOT-DEPENDENT COMPONENTS IN AON

The secondary structure of NARK includes an N-terminal signal peptide and an extracellular LRR domain, which is the proposed binding site for Q. Transmembrane and intracellular kinase domains are also key features of NARK and are essential for membrane localization, protein–protein interactions and downstream phosphorylation and signalling events. Supernodulation phenotypes result from mutations in NARK in either the LRR or kinase domains, indicating that both are required for AON signalling and/or stabilizing the signalling complex (Searle *et al.*, 2003). Modelling of the NARK LRR domain indicates that it may form a boomerang shape that acts to perceive the AON ligand (Reid *et al.*, 2011). Two known missense mutants in soybean, *nod4* and *nod3-7*, display severe supernodulation phenotypes resembling those of deletion (Men *et al.*, 2002) and nonsense (Carroll *et al.*, 1985; Searle *et al.*, 2003) mutants despite having only single amino acid substitutions within the proposed ligand-binding site (Reid *et al.*, 2011).

Mutants that affect the expression or localization of the AON receptor might also be predicted to cause supernodulation phenotypes. Shoot-controlled nodule regulation is lost in the *lss* (like-SUNN supernodulator) mutant in *M. truncatula* (Schnabel *et al.*, 2010). The *LSS* locus maps in a region close to the *SUNN* gene; however, sequencing of *SUNN* and the surrounding regions indicates that there is no mutation within the 20 kbp *SUNN* region. *SUNN* expression is greatly reduced in *lss* and epigenetic factors may be responsible for loss of SUNN activity.

As mentioned above, NARK and its orthologues share a high degree of similarity with CLV1 in arabidopsis (75 % amino acid similarity; Searle *et al.*, 2003), which is required for maintenance of the shoot apical meristem (SAM). Several protein interactions which may be relevant to the activity of NARK in AON have been reported with CLV1, including CLAVATA2 (CLV2; Jeong *et al.*, 1999) and CORYNE (CRN; Muller *et al.*, 2008). CLV2 is a receptor-like protein that lacks an intracellular kinase domain, whereas CRN is a kinase-like protein that lacks an extracellular LRR domain. CRN appears to lack effective kinase activity and may be required as a structural component in a CLV1 complex or for facilitating the inclusion of other components including CLV2 in a receptor complex (Nimchuck *et al.*, 2011).

Additional shoot-controlled supernodulation mutants have recently been genetically characterized and represent further components associated with the CLV signalling pathway (Fig. 1B, E). The shoot-dependent supernodulation mutant *sym28* in pea and *LjCLV2* in *L. japonicus* are the orthologues of *AtCLV2* (Krusell *et al.*, 2011). Experiments to determine if CLV2 forms a heterodimer complex with HAR1 in a similar manner to the CLV2–CLV1 complex in the SAM were unable to establish an interaction. KLAVIER (KLV) is a receptor-like kinase similar to RPK2/TOAD2 in arabidopsis which is required for CLV3-dependent meristem regulation (Kinoshita *et al.*, 2010; Miyazawa *et al.*, 2010). KLV was shown to form homo- or heterodimer complexes with itself and HAR1, respectively, suggesting that a receptor complex may be required for the perception of Q (Miyazawa *et al.*, 2010). This work serves to highlight the extent to which nodule regulation activity utilizes the machinery of SAM regulation. Further investigation of CLV signalling components which may function in AON will be of interest, including whether a CRN-like protein plays a role in AON.

TRANSMISSION OF AON SIGNALLING IN THE LEAF

Knowledge of signal transduction mechanisms acting downstream of CLV in arabidopsis has assisted in the identification of homologous elements in AON. Two kinase-associated

protein phosphatases (KAPP1/2) were identified in soybean that are phosphorylated by NARK *in vitro* and subsequently dephosphorylate the NARK kinase (Miyahara *et al.*, 2008). This may indicate that a sufficient equilibrium of phosphorylation states between NARK and KAPP1/2 must exist before a downstream AON response is generated (Fig. 1F).

In arabidopsis, the primary function of CLV signalling activity in the SAM is the restriction of WUSCHEL (WUS) production. This acts to maintain an appropriate balance between differentiated and undifferentiated cells via a constant feedback between WUS and CLV3 (Schoof *et al.*, 2000). *WUS-related homeobox* (*WOX*) genes have been identified in other CLE peptide/LRR receptor systems including the regulation of vascular differentiation (Hirakawa *et al.*, 2010; Ji *et al.*, 2010) and in the root apical meristem (Kamiya *et al.*, 2003; Sarkar *et al.*, 2007). Likewise, a WOX component may be involved in AON signalling, though this remains to be determined.

To identify components of AON acting downstream of NARK in the leaf, transcriptional profiling using Affymetrix GeneChips or subtractive hybridization techniques has been undertaken in soybean (Seo *et al.*, 2007; Kinkema and Gresshoff, 2008). Both of these studies identified components of the jasmonic acid (JA) biosynthesis or response pathways being regulated following rhizobia inoculation. Foliar application of methyl jasmonate in *L. japonicus* inhibited nodulation in both wild-type and *har1* plants, further indicating that JA may play a role in nodule regulation (Nakagawa and Kawaguchi, 2006).

The identification of additional nodule regulation mutants through candidate gene selection in TILLING populations or through screening of traditional mutant populations may be useful for identifying further downstream components of AON.

SDI AND EFFORTS TO IDENTIFY IT

Shoot-derived inhibitor is produced in the shoot following the perception of Q (Fig. 1G). Phloem transport of the SDI signal from the shoot to the root would appear to be the most probable mechanism based on the timing and direction of its response. It is then predicted to be perceived in the root, where it acts to prevent cell divisions required for nodule development (Fig. 1H). A bioassay approach has been exploited to characterize the nature of SDI partially through petiole feeding of plant extracts into the phloem of intact plants (Lin *et al.*, 2010, 2011). Using this technique, leaf extracts from wild-type plants inhibited nodulation in hypernodulating *nts* mutants that are unable to produce SDI. Various pre-treatments of the leaf extract showed that SDI is probably a small, heat-stable molecule that is not a protein or RNA. The inhibitory capacity of the leaf extracts was also dependent on NARK and on nod factor signalling (Lin *et al.*, 2010, 2011). The petiole feeding bioassay technique has also been exploited to show that nitrogen fixation in nodules may be systemically regulated through phloem transport of the amino acid asparagine (Sulieman *et al.*, 2010).

CONCLUSIONS AND FUTURE PROSPECTS

Plants maintain appropriate growth and development via constant feedback to environmental and internal conditions. The regulation of nodulation in legumes is one such system, where systemic signalling ensures a balance between nodule formation and energy requirements. Knowing the identity of both the root- and shoot-derived mobile signals in AON would be of immense value to the field. The identification of CLE peptides capable of nodule regulation and a role for CLV2 and KLV in AON have emphasized the similarities that exist between AON and other CLE peptide ligand–receptor systems, particularly that of the CLAVATA signalling pathway in the SAM. Ongoing research on AON will draw on these similarities and will in turn contribute to the better understanding of other environmental and developmental regulation responses occurring in the plant. The decreasing cost and increased availability of high-throughput sequencing technology continue to drive discoveries in plant genetics. Moreover, the recent sequencing of the soybean, *L. japonicus* and *M. truncatula* genomes means that three largely complete legume genomes are now publicly available (Young *et al.*, 2005; Sato *et al.*, 2008; Schmutz *et al.*, 2010). These resources will considerably support future advances in understanding the molecular mechanisms underlying nodule regulation.

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