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Minireview

Plant Hormonal Regulation of Nitrogen-Fixing Nodule Organogenesis

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Legumes have evolved symbiotic interactions with rhizobial bacteria to efficiently utilize nitrogen. Recent progress in symbiosis has revealed several key components of host plants required for nitrogen-fixing nodule organogenesis, in which complicated metabolic and signaling pathways in the host plant are reprogrammed to generate nodules in the cortex upon perception of the rhizobial Nod factor. Following the recognition of Nod factors, plant hormones are likely to be essential throughout nodule organogenesis for integration of developmental and environmental signaling cues into nodule development. Here, we review the molecular events involved in plant hormonal regulation and signaling cross-talk for nitrogen-fixing nodule development, and discuss how these signaling networks are integrated into Nod factor-mediated signaling during plant-microbe interactions.

NOD FACTOR SIGNALING DURING NODULATION

Uptake of nutrients by plants is limited by the insufficiency of biologically available forms of these compounds in the environment. As a consequence of selection pressure for survival, plants have broadly evolved beneficial symbiotic interactions with commensal microbes. The *arbuscular mycorrhizal* (AM) fungi have developed a symbiotic relationship with most land plants, which is highly beneficial for the uptake of minerals and water from the soil (Parniske, 2008). In contrast to general AM symbiosis, nitrogen-fixing nodule symbiosis has been strictly adopted by a few plant families, including legumes, together with their symbiotic partners, which are collectively called *rhizobium* bacteria (Kouchi et al., 2010; Oldroyd and Downie, 2008; Sandal et al., 2002). In general, the key signaling components for nitrogen fixing symbiosis are well conserved in the legume family (Markmann and Parniske, 2009).

To initiate symbiotic nitrogen-fixing nodulation, the host plants provide a cocktail of flavonoids to stimulate synthesis and secretion of Nod factors from symbiotic *rhizobia* bacteria (Barnett and Fisher, 2006; Perret et al., 2000). The secreted Nod factors directly stimulate their putative receptor proteins (NFRs), LjNFR1/5 (Nod factor through receptor-like kinase) in *Lotus japonicus* and MtLYK3 (LysM-RLK3)/NFP (Nod factor perception) in *Medicago trucatula*, which are peptidoglycan-binding lysine domain (LysM)-containing receptor kinases (Arrighi et al., 2006; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Recognition of Nod factors, and compatibility of *rhizobia* bacteria with host legume plants, are specifically determined by the extracellular LysM domain in the NFRs. Transgenic *M. truncatula* roots harboring LjNFR1/5 could make nodules upon inoculation of the *L. japonicus*-compatible bacterium, *Mesorhizobium loti,* and swapping the LysM between the NFRs of *L. filicaulis* and *L. japonicas* changed the ability of the plants to recognize their compatible symbiotic partners (Radutoiu et al., 2007). Activation of NFRs induced root hair deformation and subsequent stimulation of downstream signaling pathways through nuclear Ca^{2+} spiking, and induced nodule organogenesis in the inner cortex layer of roots and *rhizobia* infection by elongating infection threads from the root hair tips. A perinuclear-anchored cation channel, *MtDMI1* (*Does-not-Make-Infections1*)*/LjCASTOR/LjPOLLUX* plays a critical role upstream of Ca^{2+} spiking during early *rhizobia* infection, and its function appears to be regulated by an upstream component, *MtDMI2* (*Does-not-Make-Infections2*)*/LjSYMRK* (*Symbiosis receptor kinase*)*/MsNORK* (*Nodulation receptor kinase*), which is a member of the LRR-RLK family (Leucine rich repeat-receptor like kinases) (Catoira et al., 2000; Endre et al., 2002; Madsen et al., 2003; Radutoiu et al., 2003; Stracke et al., 2002). Loss-of-function mutations in these genes caused impaired Ca^{2+} spiking in the nucleus and nodule formation, but did not impair root hair deformation in response to *rhizobia* inoculation (Ane et al., 2004; Endre et al., 2002; Kistner et al., 2002; Stracke et al., 2002). However, both root hair deformation and Ca^{2+} spiking were disrupted in the NFR mutants, suggesting that DMI1 and DMI2 act downstream of NFRs in the Nod factor signaling pathway (Ben Amor et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Ca^{2+}-related MtDMI3 was identified as a nuclear-localized Ca^{2+}-calmodulin-dependent kinase (CCaMK) that functions downstream of Ca^{2+} spiking (Gleason et al., 2006; Tirichine et al., 2006). A *Mtdmi3* mutant could not make root nodules, even though nuclear Ca^{2+} spiking

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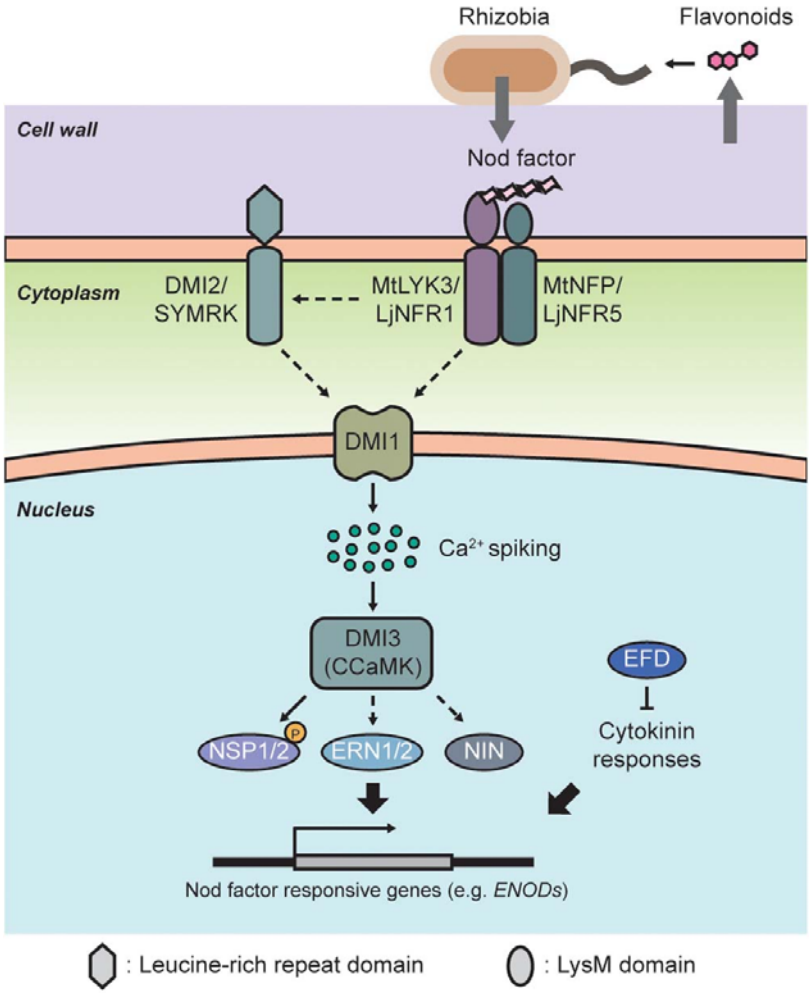

Fig. 1. Proposed model for *rhizobia*-legume symbiotic signaling pathways. Nod factor from *rhizobia* stimulates its receptor complex (NFR), MtLYK3/NFP or LjNFR1/5. Signal cascades from NFR are transduced *via* as-yet-unidentified pathways to induce nuclear calcium spiking and activate DMI3 (CCaMK). Activated DMI3 directly interacts with and activates nodule-related transcription factors, including NSP1/2, *via* phosphorylation. These transcription factors enhance the expression of Nod factor-responsive genes by directly binding to the NF-box (AATTT). It is not clear how DMI3 regulates nodule-related NIN and ERN, but their up-regulation by Nod factor signaling is essential for nodulation. EFD, an AP2/ERF family member, inhibits nodulation, probably by disrupting cytokinin signaling. The dashed arrows indicate unknown biochemical links.

was unaffected during early nodulation processes (Levy et al., 2004; Mitra et al., 2004). By contrast, constitutively activated *MtDMI3* spontaneously induced pseudo-nodules in both wild type and *Mtdmi1/2* mutants independently of upstream Nod factor signaling cues. The nuclear events of Ca^{2+} spiking and subsequent activation of CCaMK suggested that CCaMK is a regulator of transcriptional factors for early Nod factor-responsive genes such as *Expression of early nodulin* (*ENOD*s), *CYCLOPS*, and *Nodule inception* (*NIN*) (Heckmann et al., 2006; Kalo et al., 2005; Murakami et al., 2006; Smit et al., 2005; Yano et al., 2008). Plant transcriptomes are globally changed during nodule organogenesis by nodulation-related transcriptional factors including NSP1, NSP2 (Nodulation signaling pathway), ERNs [Ethylene response factor (ERF) required for nodulation], and EFD (ERF required for nodule differentiation). Indeed, MtDMI3 directly binds to the NSP1/2 complex that regulates *ENOD11* expression, which requires Nod-factor, Ca^{2+} spiking and CCaMK activity (Gleason et al., 2006; Hirsch et al., 2009; Imaizumi-Anraku et al., 2006; Tirichine et al., 2007). These results indicate that NFR-induced Ca^{2+} spiking activates nuclear localized CCaMK and then modulates transcriptional networks *via* NSP1/2 to initiate nodulation (Fig. 1).

Given the complexities of nodule development, it is not surprising that other transcription factors including ERN (ERF required for nodulation, Andriankaja et al., 2007; Middleton et al., 2007), NIN (Schauser et al., 1999), EFD (Vernie et al., 2008), and NSP1/2 are also essential for proper development of nodules. ERN1 and ERN2, which are AP2/ERF domain-containing transcriptional factors, play positive roles in nodulation. The loss-of-function mutant of *ERN1,* designated *bit1-1 (branching infection thread 1*), is impaired in early nodule formation processes, including up-regulation of *nodulin* gene expression, infection thread elongation, and nodule primordia initiation (Middleton et al., 2007). ERN3, ERN1 and ERN2 bind directly to the *cis*-element (NF-box) of the *ENOD11* promoter; however, ERN3 represses *ENOD11* expression (Andriankaja et al., 2007), suggesting that it functions as a negative regulator of nodule formation. *EFD*, another nodulation-related AP2/ERF gene, is also required for both functional bacteroid differentiation and nodule organogenesis; its loss-of-function mutant had fewer nodules defective in bacteroid differentiation. Ectopic expression of *EFD* in tobacco cells enhanced the promoter activity of type A-*MtRR4*, a negative regulator of the cytokinin signaling pathway (Vernie et al., 2008), which is involved in the regulation of nodule organogenesis. These results imply that the negative regulation of *EFD* during nodule formation might be linked to cytokinin signaling; however, more detailed studies of the biological functions of EFD are required. The putative transcription

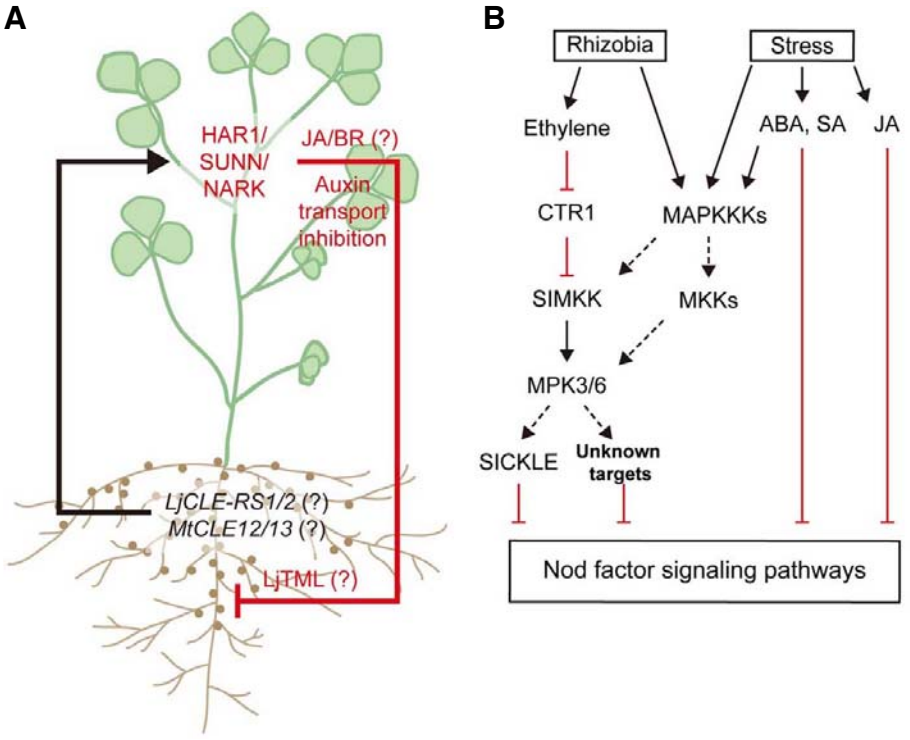

Fig. 2. Systemic and local inhibitory regulation of nodulation. (A) Schematic model of autoregulation of nodulation (AON). Long distance inhibitory pathways for AON have been proposed, but the root-derived signals are not clearly identified although some CLE peptides including LjCLE-RS1/2 or MtCLE12/13 might function as root-to-shoot signaling molecules. Root-derived signals might directly or indirectly activate the CLV1-like receptor kinase, HAR1/SUNN/NARK. These events create shoot-derived unidentified negative signals that suppress root nodule formation by inhibiting auxin transport and by presumed TML functions in the root. JA and BR are thought to be involved in shoot-derived negative signaling pathways. (B) Local inhibition pathways mediated by the coordination of stress-related hormone actions. *Rhizobia* and external stress signals activate MAPK signaling cascades and the action of plant hormones including ethylene, SA, ABA, and JA. Ethylene, a major negative regulator in nodulation, probably stimulates SIMKK(AtMKK4/5)-MPK3/6 cascades by suppressing CTR1 activity. The activated MPK3/6 proteins directly or indirectly regulate their downstream signaling components, including SICKLE/EIN2 and various stress-related transcription factors (unknown targets), to inhibit Nod factor signaling pathways. ABA, SA, and JA signaling pathways directly suppress Nod factor signaling, or their negative action could be integrated into SIMKK-mediated MAPK signaling cascades. The dashed arrows indicate unknown biochemical links.

factor, NIN, is involved at a later stage of nodulation (Borisov et al., 2003; Roussis et al., 1999; Schauser et al., 1999). A null mutation of *NIN* displayed normal epidermal responses to *rhizobia* infection, including nodule-related gene expression, Ca^{2+} spiking, and root hair deformation, but nodule organogenesis and infection thread formation were completely impaired (Borisov et al., 2003; Madsen et al., 2003). *NIN* functions downstream of *MtDMI3*, as constitutively activated CCaMK could not induce spontaneous nodule formation in an *Mtnin* loss-of-function mutant (Marsh et al., 2007). The Nod-signaling cascade initiated from NFRs is linked to nuclear CCaMK activity, which regulates nodule-related transcription factors during initiation of nodulation at the epidermis (Fig. 1). However, the upstream signaling component(s) that are linked to phosphorylation-dependent activation of nodulation transcription factors remain to be elucidated (Fig. 1).

SYSTEMIC AUTOREGULATION OF NODULATION

Negative regulation of nodulation by systemic shoot-root communication

During nitrogen-fixing symbiosis, the number of nodules has to be strictly regulated because nodule development is an energetically expensive process for the leguminous host plants. The symbiotic relationship is formed by the legumes obtaining nitrogen from the endophytic bacteroids, while providing photosynthetic products in return. Therefore, the maintenance of a balanced number of nitrogen-fixing nodules is critical to provide optimum nutrition and prevent excessive energy drains on the host plants. To control the number of nodules, legumes have evolved negative feedback systems, such as systemic autoregulation of nodulation (AON) and local hormonal inhibitory regulation (Figs. 2A and 2B).

Systemic autoregulation of nodulation in legumes was proposed by Nutman in 1952 after observing a transient increase in the number of new nodules in red clover roots after excising old nodules (Nutman, 1952). The molecular mechanism of AON was actively investigated after identification of the supernodulation mutants, *hyper nodulation and aberrant root 1 (har1), super numeric nodules 1 (sunn)*, and *nodule autoregulation receptor kinase (nark)* in *L. japonicus, Medicago truncatula*, and *Glycine max*, respectively (Ferguson et al., 2010; Kawaguchi and Minamisawa, 2010; Krusell et al., 2002; Nishimura et al., 2002; Oka-Kira et al., 2005; Searle et al., 2003), which encode a leucine rich repeat (LRR) CLV1 (CLAVATA)-like receptor-like kinase, and were exclusively expressed in the phloem of shoot tissues and root nodules (Nontachaiyapoom et al., 2007). Interestingly, reciprocal grafting assays between supernodulation mutants and wild-type plants showed that the supernodulation phenotype is determined by the shoot genotype (Krusell et al., 2002; Lough and Lucas, 2006; Ruiz-Medrano et al., 2001). These observations, along with the molecular mechanism of CLV1 (CLAVATA 1) triggered by a signal peptide CLV3 (CLAVATA 3), led to the hypothesis that communication between roots and shoots *via* a peptide-mediated mobile signal is likely involved in the systemic AON. Of the 39 potential *CLE* (*CLV3/ESR-related*) genes that have been identified in the *L. japonicus* genome, *LjCLE-RS1/2* appeared to function as a root-derived mobile

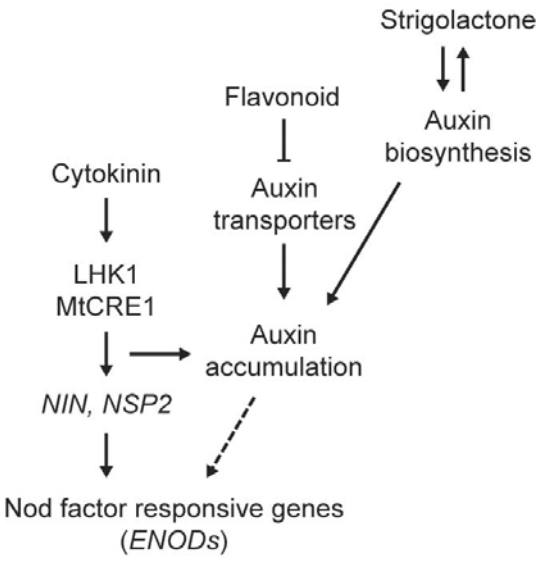

Fig. 3. Positive regulation of nodulation through the action of plant hormones. The interplay of auxin and cytokinin plays a major role in nodule organogenesis. Cytokinins positively regulate nodule-related *NSP2* and *NIN* expression, and expression of *PINs* that lead to local auxin accumulation. The local accumulation of auxin during the initiation of nodule primordia is also promoted by cooperation between flavonoids and strigolactone. The dashed arrows indicate unknown biochemical links.

signal for AON because: 1) expression levels were rapidly up-regulated by *rhizobia* inoculation or KNO_3 treatment; and 2) overexpression significantly suppressed nodulation in wild type plants, but not in a loss-of-function *har1* mutant (Okamoto et al., 2009). It is likely that MtCLE12/13 in *M. truncatula* plays a similar role to that of LjCLE-RS1/2 (Mortier et al., 2010). These CLE peptides might be processed to functionally active forms in the root and transported to shoot tissues (*via* the phloem) where they subsequently stimulate AON-related LRR-RLKs, such as HAR1 and its orthologs (Fig. 2A). However, this hypothesis remains to be tested. The shoot-derived negative signals from the LRR-RLKs in AON are presumably delivered to roots where they are decoded to optimize nodule numbers. An as-yet-uncloned gene, *LjTML* (*Too much love*), was identified as a potential signaling decoder for shoot-derived signals in AON (Magori et al., 2009). The *tml* mutant exhibited a supernodulation phenotype, which was determined by the root genotype. Moreover, the nodule phenotype of *tml* was less sensitive to exogenous nitrogen treatment, and this decreased sensitivity was not enhanced by the *har1* mutation. These results indicate that TML is one of the AON signaling components that function downstream of HAR1 in root tissues; however, further experiments are required to elucidate the detailed mechanism of its action in AON (Fig. 2A).

Plant hormones as candidates for shoot-derived signals in AON

The plant hormones auxin, jasmonic acid (JA), and brassinosteroids (BR) appear to act as signaling mediators in the AON signaling pathway (Nakagawa and Kawaguchi, 2006; Oka-Kira and Kawaguchi, 2006; Terakado et al., 2006). Foliar application of JA decreased the nodule number in AON-defective hypernodulation mutants of *G. max* and *L. japonicus* (Kinkema and Gresshoff, 2008; Nakagawa and Kawaguchi, 2006). JA biosynthesis in the foliar region of soybean was enhanced by inoculation of symbiotic *rhizobia,* but this increase was impaired in AON-deficient *Gmnark* mutants (Kinkema and Gresshoff, 2008), suggesting that *rhizobia* bacteria-mediated activation of JA biosynthesis is controlled by a GmNARK-mediated AON signaling pathway.

Exogenous BR treatment effectively reduced the number of nodules in a soybean hypernodulation mutant, but not in wild type plants. Exogenous BR also enhanced the biosynthesis of spermidine (Terakado et al., 2006), which caused a decrease in the number of nodules. Thus, negative regulation of nodulation by BR might be mediated through the action of spermidine in shoots. In spite of the immobility of BR in the pea (*Pisum sativum*) (Symons and Reid, 2004), defective nodulation in a BR-deficient pea mutant was recovered in wild-type shoot grafting experiments, implying that BRs might regulate shoot-derived AON signaling *via* unknown pathways (Ferguson et al., 2005). It is clear that BR play a critical role in AON; however, the discrepancy between the negative effects of exogenous BR on nodulation and impaired nodule formation in BR-biosynthetic or BR-insensitive pea mutants suggests that BR might differentially control nodulation with respect to local and systemic regulation. Indeed, BR facilitates polar auxin transport by enhancing *PIN* expression in *Arabidopsis* (Bao et al., 2004; Nakamura et al., 2004), and auxin transport and its subsequent local accumulation are important in nodule organogenesis (Pacios-Bras et al., 2003; van Noorden et al., 2006; 2007). These results lead to a possibility that lower auxin levels in the roots of BR-deficient legumes decrease the initiation of nodule primordia, although BR-mediated AON signaling from the shoot might be defective.

Polar auxin transport also appears to be important in AON signaling pathways (van Noorden et al., 2006; Wasson et al., 2006). The inoculation of *rhizobia* normally decreased auxin loading from shoots to roots by increasing the level of flavonoids, inhibitors of auxin transport, in the shoot. However, the hypernodulation *Mtsunn*, a loss-of-function mutant of AON-related LRR-RLK in *M. truncatula,* showed much higher auxin transport than the wild type, and *rhizobia* inoculation could not reduce the polar auxin transport to root tissues in this mutant (Penmetsa et al., 2008). These results support a hypothesis that AON signaling pathways could inhibit acropetal polar auxin transport; thus, decreased auxin levels in root tissues could inhibit the development of newly initiated nodule primordia (Fig. 2A).

LOCAL REGULATION OF NODULATION *VIA* HORMONES

Ethylene, a major local inhibitory signal in symbiosis

Legumes have evolved local inhibitory regulation systems of nodulation *via* hormones such as Abscisic acid (ABA), Jasmonic acid (JA), ethylene, and SA (Salicylic acid), in addition to systemic AON signaling (Biswas et al., 2009; Ding et al., 2008; Oldroyd et al., 2001; Penmetsa and Cook, 1997; Sun et al., 2006; Suzuki et al., 2004) (Fig. 2B). The hypernodulating *sickle* (*sik*) mutant of *M. truncatula* was caused by a loss-of-function mutation (Q893stop) in the ortholog of *Arabidopsis Ethylene insensitive 2 (EIN2)*, a key component of the ethylene signal transduction pathway (Penmetsa and Cook, 1997). The *sickle* mutant was defective in ethylene responses and showed increases in nodule numbers by generating pairs of nodules at opposite regions of both the xylem and phloem (Penmetsa and Cook, 1997; Penmetsa et al., 2008). Furthermore, numerous

uncontrolled infection threads and root hair hypersensitivity to Nod factors were observed in the *Mtsickle* mutant. This genetic evidence of the negative role of ethylene was supported by the effects of an ethylene precursor, Aminocyclopropane-1-carboxylic acid (ACC), and an ethylene biosynthetic inhibitor, AVG (Aminoethoxyvinylglycine), on nodulation (Engstrom et al., 2001; Oldroyd et al., 2001). ACC or ethylene treatments strongly suppressed the nodulation and Nod factor signaling pathways, but inhibition of ethylene signaling or biosynthesis significantly increased the nodule numbers. Moreover, ethylene treatment effectively inhibited Nod factor-inducible Ca^{2+} spiking and the expression of nodule-related *ENOD*s (Engstrom et al., 2001; Oldroyd et al., 2001; Penmetsa and Cook, 1997), supporting the evidence for a negative effect of ethylene on nodulation by disrupting Nod factor signal transduction pathways. Ethylene inhibits nodulation by altering the nuclear Ca^{2+} spiking frequency and suppressing nodulin gene expression, suggesting that EIN2-mediated ethylene signaling and Nod factor signaling pathways are probably integrated downstream of CCaMK signaling. The elucidation of how ethylene signaling pathways regulate calcium spiking during early nodulation will provide critical clues to understand ethylene-mediated local inhibition of nodulation.

Stress-related JA, SA, and ABA signaling inhibits nodulation

Stress-related hormones such as JA and ABA greatly reduce nodulation by disrupting Nod factor-induced Ca^{2+} spiking and nodulation-related gene expression (Akune et al., 2004; Cardoza et al., 2006; Ding et al., 2008; Stacey et al., 2006; Sun et al., 2006; Suzuki et al., 2001). JA-treated *Medicago* roots rarely induced symbiotic nodules and consistently showed reduced expression of *ENOD11* and *RIP1* (*Rhizobium meliloti-induced peroxidase gene*). However, these JA effects were suppressed by an ethylene biosynthetic inhibitor, AVG, and the *sickle* mutant was less sensitive to exogenous JA in nodulation (Sun et al., 2006). These results imply that JA-mediated inhibition of nodulation is linked to ethylene signaling pathways. This hypothesis is supported by the previous results that JA and ethylene share downstream target genes, including *ERF1* and *PDF*s, which function to stimulate plant defenses and repel insects, respectively (Lorenzo et al., 2003). Similar to JA, ABA decreased nodule numbers and disrupted Nod factor-induced calcium spiking (Ding et al., 2008). ABA-insensitive *Medicago* roots were generated by overexpressing *Arabidopsis abi1-1,* an ABA-insensitive dominant allele of *ABI1* (*ABA insensitive 1*), and these roots formed vigorous nitrogen-fixing nodules. However, the *sickle* mutation could not suppress the negative effect of ABA on nodule formation. Furthermore, ABA repressed the expression of a different set of nodulation-related genes, including cytokinin-responsive *ENOD40* and *NIN*. It is clear that ABA negatively regulates nodulation *via* a different regulatory mechanism from JA.

Nodulation and *rhizobia* infection in legumes was promoted by reduction of the endogenous SA level by ectopic expression of a bacterial *nahG* gene, which encodes an SA hydroxylase (Stacey et al., 2006). Exogenous SA represses the expression of nodule-related genes and nodule development, but it also suppresses the growth of *rhizobia* (Stacey et al., 2006). SA is a key signaling molecule for defense responses to diverse plant invaders. Recent studies show that defense and stress responses are elicited during *rhizobia* infection, and these responses are important factors for determining host range or nodule numbers (Mellor and Collinge, 1995; Santos et al., 2001). However, the molecular mechanisms that mediate SA-induced defense signaling during nodulation are largely unknown. In the future, the precise roles of SA and SA-mediated defense signaling during nodulation should be investigated.

Are local inhibitory hormone signals integrated into MAPK cascades?

Constitutive triple response 1 (CTR1) MAPKKK-mediated MAPK (Mitogen activated protein kinase) signaling modules act as major signaling components in ethylene signal transduction (Kieber et al., 1993). A gain-of-function mutant in *Arabidopsis*, *ctr1*, exhibited a constitutively activated ethylene-related triple response, and ethylene or ACC treatment activated two specific MAPK proteins in plants (Cardinale et al., 2002; Yoo et al., 2008). In *Medicago sativa*, specific MAPKs, such as MMK3 (AtMPK13), SAMK (Stress activated MAPK, AtMPK3), and SIMK (Salt stress induced MAPK, AtMPK6), were rapidly activated by ACC treatment *via* SIMKK (Salt stress induced MAPKK, AtMKK4/5) (Cardinale et al., 2002; Kiegerl et al., 2000). Overexpression of SIMKK phenocopied the *ctr1*-like constitutive ethylene response phenotype and enhanced the expression of ethylene-responsive genes. Furthermore, the SIMKK (AtMKK4/5)-SIMK (AtMPK6) module also plays an essential role in ethylene biosynthesis and stress responses in other plants (Kim et al., 2003; Liu and Zhang, 2004). It is therefore likely that CTR1-mediated MAPK signaling cascades mediate the local inhibitory regulation of nodulation during ethylene signaling in legumes (Fig. 2B). In addition to ethylene signaling, MAPK signaling cascades are implicated in MAMP (Microbial associated molecular pattern)-triggered immunity (MTI) and diverse stress responses in plants (Asai et al., 2002). Inoculation of symbiotic *rhizobia* could effectively activate MPK3/6 and subsequently enhance the expression of defense/stress-related genes in legumes (Duzan et al., 2004; Lopez-Gomez et al., 2011). Ethylene biosynthesis was also increased in response to *rhizobia* inoculation in *L. japonicus* (Lopez-Gomezet al., 2011; Sandal et al., 2002), but these defense/stress-related responses rapidly disappeared during nodulation (Lohar et al., 2006). However, inoculation of non-compatible *rhizobia* strains, or a *rhizobia nodC* mutant that is impaired in Nod factor biosynthesis, induces SA accumulation and diverse stress responses in legume roots (Stacey et al., 2006). It is possible that the symbiotic *rhizobia* could induce plant defense responses through MAPK cascades or ethylene signaling at the initial stage of interaction, but unidentified signals from the symbiotic bacteria or plant-microbe interactions might suppress the plant defense response to allow invasion of the *rhizobia*. Consistent with this idea, MAPK signaling stimulators, such as abiotic stresses, and a bacterial MAMP, flg22, greatly reduced nodule formation (Duzan et al., 2004; Lopez-Gomez et al., 2011; Sandal et al., 2002). A recent report shows that LjSYMRK directly interacts with SymRK-interacting protein 2 (SIP2), a SIMKK ortholog, and inhibits the kinase activity of SIP2 for MPK6 activation in *L. japonicus* (Chen et al., 2012). SIP2 and its homologous MKKs are central signaling components for defense, ethylene responses, and abiotic stress responses (Ronald and Beutler, 2010; Sinha et al., 2011; Zhang and Klessig, 2001). Together, SYMRK-mediated Nod factor signaling might suppress diverse defense and stress responses induced by the SIP2-mediated MAPK signaling cascade upon *rhizobia* infection; however, *SIP2* knockdown transgenic hairy roots were defective in nodulation and infection thread formation, which indicates that SIP2 acts as a positive regulator of nodulation. A gain-of-function study of MAPK cascades during nodulation could elucidate the

role of MAPK signaling and cross-talk with Nod factor signaling.

Ethylene, JA, SA, and ABA are involved in plant responses to pathogens, wounding, and various abiotic stresses, and are known to stimulate MAPK signaling cascades. In turn, MAPK signaling pathways are probably coordinated with Nod factor signaling in legumes to optimize nodulation during the symbiotic interaction (Fig. 2B). More detailed studies on signaling interplays of stress-related hormones, MAPKs and Nod factors will be necessary to understand the negative regulation of nodulation.

Positive roles for hormonal regulation of nodulation: the essential roles of cytokinin in nodule organogenesis

As we expected, given the complexity of signlaing networks in nodule organogenesis, not only negative action in nitorgen-fixing nodulation, but some phytohormones including cytokinins, auxin, GAs, BRs and strigolactone are also positively involved in the nodule organogenesis and development.

Cytokinin is a key signaling molecule for nodule organogenesis in symbiotic interactions between leguminous plants and *rhizobia*. The earliest evidence for cytokinin action in nodulation was the morphogenic rescue of nodule formation in *Medicago sativa* with nonsymbiotic bacteria carrying the *isopentenyl transferase* (*IPT*) gene of *Agrobacterium tumefaciens* (Cooper and Long, 1994). Moreover, a cytokinin primary responsive type A-*RR* (*response regulator*) is activated in nodule primordial cells in the cortex layer during nodulation, and overexpression of cytokinin-degrading *cytokinin oxidase* (*CKX*) decreases nodule organogenesis in *L. japonicus* (Lohar et al., 2004). Silencing *3-hydroxy-3-methylglutaryl CoA reductase 1* (*HMGR1*), a gene encoding an enzyme involved in the synthesis of the cytokinin precursor isoprenoid, reduces the number of nodules in *Medicago truncatula* roots (Kevei et al., 2007).

Suppression of *MtCRE1*, a highly conserved gene for the *Arabidopsis* cytokinin receptor *Cytokinin resistant 1 (CRE1)/ Arabidopsis histidine kinase 4 (AHK4)*, resulted in severely defective nodulation in *Medicago truncatula* (Gonzalez-Rizzo et al., 2006). Loss-of-function *Lotus histidine kinase 1 (LHK1)*, a cytokinin receptor of *L. japonicus*, designated as *hit1,* completely abolished nodule primodial development, but did not affect infection thread formation enabling *rhizobia* invasion (Murray et al., 2007). By contrast, *Spontaneous nodule formation 2* (*snf2*), a gain-of-function mutant of *LHK1,* spontaneously developed root nodules in spite of the absence of *rhizobia* inoculation, and it exhibited constitutively activated cytokinin responses (Tirichine et al., 2007). The activation of cytokinin signaling is deemed as necessary to initiate nodule organogenesis.

Cytokinin signaling is integrated into Nod factor signaling pathways

The manner in which cytokinin and Nod factor signaling pathways are integrated for nodule development has been the subject of considerable research. The expression of a critical nodulation gene, *NIN,* was induced by both cytokinin and Nod factor perception (Gonzalez-Rizzo et al., 2006; Murray et al., 2007), but its expression was completely blocked in the loss-of-function mutants *Mtcre1* and *Ljhit1* (Plet et al., 2011), whereas expression was up-regulated in the constitutively activated *snf2* mutant (Murray et al., 2007; Plet et al., 2011; Tirichine et al., 2007; Wasson et al., 2011). Furthermore, the *snf2* mutation rescued the aborted nodulation phenotype of loss-of-function mutants of Nod factor receptors, *nfr1* and *nfr5,* and a symbiosis related LRR-RLK mutant, *symrk*. However, *snf2* could not rescue the impaired nodulation phenotype of the *nin* and *nsp2* loss-of-function mutants. Therefore, cytokinin signaling is likely to be regulated by NFRs and integrated into transcriptional regulation of *NIN* and *NSP2* during nodule organogenesis (Sandal et al., 2007; Tirichine et al., 2007). The long-distance crosstalk networks between the infection signaling pathways in the epidermis and the cytokinin-mediated organogenic pathways in the cortex still remain to be elucidated.

Cytokinin signaling interacts with other hormones such as ethylene and auxin during nodule organogensis. The *skl/cre1* double mutant reduced root nodule numbers compared with the wild type, but had more nodules than *cre1* (Plet et al., 2011; Wasson et al., 2011). The *cre1* mutation restored the *skl* irregular nodulation pattern to the wild-type nodulation pattern. Cytokinin signaling functions in parallel with ethylene-mediated local inhibitory signaling pathways to control nodule numbers, but integrates with ethylene signaling to determine the pattern of nodule formation. Interestingly, MtCRE1-mediated cytokinin signaling induced the expression of a subset of *MtPINs*, leading to local auxin accumulation during initiation of nodule primodia (Plet et al., 2011; Wasson et al., 2011). Nod factors also induced auxin accumulation by inhibiting polar auxin transport in dividing pericycle and cortex cells, which is critical for nodule initiation (Hirsch et al., 1989; Pellerone et al., 2006; Plet et al., 2011; Wasson et al., 2011). Although it is still unclear whether Nod factors or flavonoids induced by the bacteria control cytokinin signaling for auxin accumulation, local auxin accumulation induced by cytokinin in primordial dividing cells is a critical event for nodule organogenesis (Fig. 3).

Local Auxin accumulation is important for nodule organogenesis

During plant organogenesis, the balance of auxin and cytokinin signaling is critical for generating secondary organs and maintaining the meristematic activity of plant stem cells (Stahl and Simon, 2010). The tight regulation of auxin accumulation is necessary and sufficient to initiate organ formation (Benkova et al., 2003). The first evidence of auxin-dependent nodule organogenesis in legumes was the observation that auxin transport inhibitors could induce pseudo-nodule formation in *Medicago sativa* without *rhizobia* or Nod factor inoculation, and auxin transport inhibitors also activated the expression of early nodule-related *ENOD2* (Bhuvaneswari et al., 1989; Hirsch et al., 1989). Activation of the auxin-responsive reporter, *GH3*, was observed in nodule primordial cells during symbiotic interaction with *rhizobia* (Mathesius et al., 1998). Furthermore, inoculation of *rhizobia* harboring an auxin biosynthetic enzyme increased the nodule number compared to inoculation of wild-type *rhizobia* (Pii et al., 2007). Therefore, it seems that local auxin accumulation is a prerequisite for nodule primordia initiation, and auxin signaling pathways function in parallel, or downstream of, the Nod factor-mediated signaling pathway.

Symbiotic *rhizobia* dependent flavonoid production in legumes is a primary regulatory step for local auxin accumulation during early nodulation processes. A molecular mechanism underlying flavonoid-mediated auxin accumulation and nodule formation has been proposed in a genetic study of *Arabidopsis* (Peer et al., 2004; Taylor and Grotewold, 2005). Flavonoid biosynthetic mutants exhibited an increased acropetal transport of auxin in roots compared with the wild type, and auxin transport was completely blocked by exogenous application of flavonoids in *Arabidopsis*. Flavonoids could bind to the auxin transporters AtMDR (Multi-drug resistance), AtAPM (Aminopeptidase M1), and AtPINs (Pin-formed) (Peer et al., 2004), and prevent their intracellular trafficking (Taylor and Grotewold,

2005), suggesting that *rhizobia*-induced flavonoids might regulate local auxin accumulation in legume roots in early nodulation. Silencing *MtCHS* (*Chalcone synthase*) and *GmIFS* (*Isoflavone synthase*) that encode key enzymes for flavonoid production dramatically inhibited nodulation in host legume plants (Pellerone et al., 2006; Subramanian et al., 2006; Wasson et al., 2006). The inhibition of polar auxin transport by *rhizobia*-mediated flavonoid biosynthesis and the subsequent accumulation of auxin in nodule initiation sites are early physiological events that induce cell differentiation in nodule primordia (Fig. 3). Despite the critical roles of auxin in plant organogenesis and differentiation, the way in which auxin signaling and local accumulation are integrated into Nod factor-dependent signaling modules is still largely unknown. The characterization of functional auxin transporters and signaling elements that induce local auxin accumulation during nodule initiation would help to decipher how auxin signaling regulates nitrogen fixing nodule development with diverse signal cues.

Gibberellin and strigolactone regulate nodule development

Gibberellins (GAs) are a class of growth-promoting hormones in plants. Early studies suggested that GAs play a positive role in nodulation. GAs, along with the biosynthetic oxidase gene, *GA20,* accumulate in developing nodules, bacterial invasion tracks, and pre-infection zones in the lima bean (*Phaseolus lunatus*) and cowpea (*Vigna unguiculata*) (Dobert et al., 1992; Lievens et al., 2005). Application of GA biosynthesis inhibitors causes severe nodulation defects in Sesbania (*Sesbania rostrata)* (Lievens et al., 2005). A GA-deficient pea mutant also showed decreased nodule numbers (Ferguson et al., 2005; Ross et al., 2005). However, recent genetic and pharmacological studies of GA during nodulation have revealed a negative effect of GA on nodule development by *L. japonicus*. Both overexpression and gain-of-function mutations of *LjSLEEPY*1, a positive regulator of GA signaling, significantly reduced nodule numbers (Maekawa et al., 2009). The root hair curling triggered by *rhizobia* was clearly inhibited by exogenous GA treatment. Furthermore, GA treatment decreased the expression of *NSP2* and *NIN* during early nodulation processes. These results demonstrate that GA negatively regulates nodule formation by interfering with nodulation signaling pathways (Maekawa et al., 2009). These contradictory observations regarding GA effects indicate that there are some evolutionary differences (or alternative GA signaling networks) in nodulation in different legume species.

Strigolactone was initially identified as a host recognition signal for *mycorrhizal* fungi in AM symbiotic interactions (Yoneyama et al., 2008), and its biological role has been recently defined as that of a novel plant hormone involved in the inhibition of shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008). Strigolactone also affects nodulation (Foo and Davies, 2011; Soto et al., 2010). Exogenous application of GR24, a synthetic strigolactone, increased the number of nodules in *Medicago sativa,* but did not affect symbiotic bacterial growth and flavonoid biosynthesis (Soto et al., 2010). The strigolactone-deficient pea *rms1* mutant showed a significantly decreased number of nodules, and exogenous treatment with GR24 rescued the defects of nodule development in *rms1* (Foo and Davies, 2011). Therefore, it is now evident that strigolactone acts as a positive regulator of nodule development; however, the molecular mechanisms underlying strigolactone action in nodulation are still obscure. A recent report shows that low nitrogen or phosphate conditions in soil stimulate strigolactone biosynthesis in plants (Xie et al., 2010). Strigolactone also promotes auxin biosynthesis and modulates auxin and cytokinin signaling pathways (Ferguson and Beveridge, 2009; Xie et al., 2010; Yoneyama et al., 2010). The availability of nitrogen in the rhizosphere is one of the main factors determining nodule numbers, and auxin and cytokinin are positively involved in nodule development. These observations lead us to speculate that the strigolactone biosynthesis and signaling pathways are closely connected to nutritional conditions, whereas other plant hormones control nodule numbers (Fig. 3).

CONCLUSIONS

Nodulation is strictly controlled by the host plant to ensure appropriate levels of nitrogen fixation without excessive depletion of photosynthetic products. The host leguminous plants negotiate with symbiotic bacterial partners to optimize the levels of nodulation during nitrogen fixing symbiotic interactions. The coordination of plant hormones is broadly and tightly associated with the control of nodule formation, which likely co-evolved with symbiotic partners to adjust the availability of nitrogen and to reduce the carbon drain to a minimum. Because sessile land plants strictly regulate their resources in response to environmental changes, it is reasonable to postulate that legumes utilize stress- and growth-related hormones as developmental cues to regulate nodulation.

It is clear that the coordination of hormonal signaling pathways is essential for nodule organogenesis. However, the mechanisms of spatio-temporal cooperative regulation between Nod factor and hormone signaling pathways during symbiotic interactions are not clear. Although all legumes can produce nitrogen-fixing nodules with their symbiotic partners, the efficiency of nitrogen fixation can differ by more than 10-fold between legume species (Den Herder and Parniske, 2009). Regulation of nodule number is one of the critical factors causing this large difference in nitrogen fixing efficiency. Recently, the overuse of nitrogen fertilizers has caused many serious global problems such as soil acidification and dead zones in the ocean caused by overgrowth of algae. Understanding the mechanics and regulation of nodulation, and translating this knowledge to optimize nodule development through agricultural biotechnology, will enable significant progress toward resolving these global issues.

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